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K. O. Renkonen

ON THE 50TH BIRTHDAY OF

PROFESSOR

K. O. RENKONEN

Karl Olof Renkonen, M.D., Professor of Bacteriology and Serology at the University of Helsinki, will reach the age of 50 years on January 25th, 1955.

On this happy occasion his numerous friends and pupils wish to give expression to the gratitude and attachment felt towards him. The appreciation is deep especially for the time and energy which he has devoted, to an extent unusual under the conditions prevailing in Finland, to the training of young scientists, and for the support which he has given to them from his extensive store of knowledge and his genial and warm personality.

Preparatory to entering the University of Helsinki, Renkonen graduated from the Suomalainen Normaalilyseo, Helsinki, in 1923. In 1926 he took the degree of Candidate of Philosophy, majoring in chemistry. The principal stages in his career since then have been as follows: Candidate of Medicine in 1927, Licenciate of Medicine in 1933, Doctor of Medicine and Surgery in 1935, University Docent in Bacteriology and Serology in 1937, Diploma in Internal Medicine in 1938, Professor of Bacteriology and Serology in 1942. In 1943—1946, in addition to holding the university chair, he was the temporary chief of the State Serum Institute.

His training and experience in chemistry and internal medicine have been supplemented by other positions held by him: those of amanuensis in the Department of Medical Chemistry of the University of Helsinki in 1930—1934, assistant instructor in the same Department in 1934—1936, assistant laboratory physician in the Maria Hospital, Helsinki, in 1933—1936, and chief of laboratory in

the Mehiläinen Hospital in 1937—1939. Among his numerous travels abroad for purposes of study may be mentioned a year spent in the Pasteur Institute, Paris, in 1938—1939 on a Rockefeller scholarship and a four months' tour in the United States in 1947.

During the Finnish-Russian Winter War of 1939—1940 Renkonen served as chief of a field laboratory, and in the war of 1941—1944 as head of the Army Blood Transfusion Service. In the performance of these duties he manifested his great powers of initiative and his intrepidity as a soldier whose resources never failed even under the most difficult circumstances. He was promoted to the rank of Major in the Medical Corps in 1940 and that of Lieutenant Colonel in 1944.

Professor Renkonen is known to the students of Medicine as an excellent, lively and inspiring teacher whose lectures never are dull, at the same time as there is a feeling of assurance that the knowledge imparted by the professor is definitely up to date and even more recent than that obtainable from the latest edition of the textbook.

Renkonen's own contributions to scientific literature reveal a worker in many branches of the medical field, who is capable of dealing in a critical and characteristic manner with a variety of subjects in practical medicine as well as with questions relating to the improvement of our basic theoretic knowledge. He has commendably augmented the heritage of his father in the field of blood group investigation by adding, as his own accomplishment, a series of papers on group-specific agglutinins occurring in plants in order to shed light on the fundamental nature of blood group characteristics also from this aspect.

So far Renkonen has occupied the professor's chair for only ten years under peacetime conditions. Postwar economic difficulties in Finland have rendered it impossible for the Department of Serology and Bacteriology to obtain all the material facilities required for good working conditions. Despite this drawback, 33 doctoral theses and a large number — over 200 — other reports of investigations have been published by this Department during the time of his directorship. The carrying out of such a remarkable amount of work in the limited premises has been possible only through Renkonen's greatly encouraging attitude toward the work of his students.

The genuine erudition which is characteristic of Renkonen — a happy combination of intelligence, imagination and constant reading — forms an invaluable support which contributes to the success of the work of his pupils. Nevertheless, for the pupil personally it has frequently been of even greater importance that from the very outset he has been able to look up to Professor Renkonen with the confidence of finding in him a fellow human being and a friend, who is ready and able to say an encouraging word at the right time.

In honour of Professor Renkonen's birthday, fifty-seven of his friends and pupils have submitted manuscripts of scientific studies, for compilation into a volume to be presented to him in commemoration of this anniversary.

T. W. Wartiovaara

FROM THE MICROBIOLOGICAL LABORATORY, LÄÄKETEHDAS ORION OY, HELSINKI

EFFECT OF ALUMINIUM PHOSPHATE AND ALUMINIUM HYDROXIDE ON THE ANTIGENICITY OF PURIFIED DIPHTHERIA TOXOID

by

PEKKA HALONEN and LAURI JÄNNES

(Received for publication February 4, 1954)

In the immunization of children against diphtheria, prophylactics wherein the toxoid is adsorbed on a mineral carrier are now in general use. In several Central European countries and in Denmark the diphtheria toxoid in the final stage of preparation is adsorbed on aluminium hydroxide [Willstätter Cγ-modification (7)]. In England, Sweden and Finland aluminium phosphate gel is used as the adsorbent. The new developments, the protamine diphteria toxoid of Ross (6) and the natural adjuvant prophylactic of Lahiri (3), are not yet tested conclusively.

Very little comparative information about the respective antigenicities of aluminium phosphate (AP) and aluminium hydroxide (AH) prophylactics seems to be available. The purification procedures and the purity levels of diphtheria toxoid vary considerably in different preparations. Likewise, the methods used in the evaluation of the potency of diphtheria prophylactics are not yet universally standardized. For instance, in Germany, the antigenicity of diphtheria prophylactics is determined as protective units (Schutzeinheiten), according to the method of Prigge (4). In many other countries, the antitoxin formation in immunized test animals is estimated. Consequently, until an international reference standard for diphtheria prophylactics is available, a comparison of the anti-

genicities of different preparations cannot be obtained from the published literature.

Both adjuvants AH and AP have their advantages and disadvantages. Both considerably enhance the antigenicity of diphtheria toxoid. The Cγ-modification of AH is a more powerful adsorbent than AP. Less AH is needed for the complete adsorption, and this can be carried out at pH 6.5; thus, a subsequent alteration of the pH in the final sterile prophylactic is unnecessary. On the other hand, the preparation of constantly similar lots of the gel is relatively difficult and requires production on rather a large scale. The combination of the toxoid with AH is so firm that an evaluation of the toxoid content of the final prophylactic with the flocculation technique causes difficulties.

An efficient AP gel is easy to prepare, but a rather large quantity of it is needed for adequate adsorption. The optimum pH for the adsorption is 5.5; thus adjustment of the pH to 6.5 after a prolonged adsorption time is needed to avoid unnecessary irritation in vaccination and to ensure good stability for the toxoid. Alteration of pH in solutions which cannot be sterilized afterwards is always a somewhat troublesome procedure.

A comparison of these two types of diphtheria prophylactics thus seemed to be well worth while. Consequently, we prepared from the same batch of purified diphtheria toxoid both an AP and an AH adsorbed preparation. During a period of one year, their respective immunizing potency was followed by estimating the antitoxin formation in immunized guinea pigs.

MATERIALS AND METHODS

Materials. — The diphtheria toxoid was the purified diphtheria toxoid used routinely in this laboratory for the preparation of diphtheria and combined prophylactics. The toxin is produced using the substrain CN2000 of Corynebacterium diphtheriae P.W. No. 8.[1] and a modification of the semisynthetic casein hydrolysate medium developed by Holt (1). The toxoidization is carried out according to Holt. In the purification, an ammonium sulphate fractionation followed by a decolourization with activated charcoal is employed. The toxoid batch used in this investigation had the following characteristics: Purity, 1700 Lf doses/ml; flocculation time, (Kf), 10 min. at 50 Lf level, and concentration, 750 Lf doses/ml.

[1] This strain was obtained from Wellcome Research Laboratories, Beckenham, England, through the kindness of Dr. F. V. Linggood.

Aluminium phosphate gel (AP) was prepared according to Holt (2).

Aluminium hydroxide gel (AH) was a preparation of the Willstätter $C\gamma$-modification, which Dr. H. Eibl from the Bundesstaatliches Serotherapeutisches Institut, Vienna, Austria, kindly placed at our disposal.

AP prophylactic, which closely simulates Holt's P.T.A.P. (2), was prepared to contain 60 Lf doses of the toxoid and 10 mg of $ALPO_4$/ml in physiological saline solution; the preservative was 0.01 per cent Merthiolate (Eli Lilly & Co). The pH was adjusted to 5.5. After storage for one year at +4° C, 15 Lf doses were found to be free in solution.

AH prophylactic was prepared to contain 60 Lf doses of the toxoid and 2.5 mg of $Al(OH)_3$/ml in physiological saline solution; the preservative was 0.01 per cent Merthiolate. The pH was adjusted to 6.5. After storage for one year, no toxoid was found to be free in solution.

Determination of Antigenicity. — Immunization: Guinea pigs weighing 400 to 450 g were immunized with two successive injections of diluted prophylactic. The diluent was 0.9 per cent NaCl. The interval between the injections was four weeks.

Determination of antitoxin: The antitoxin formation in the serum of individual immunized guinea pigs was estimated, with the use of the intracutaneous guinea pig method of Römer (5), at the 1/100 IU/ml level. Each serum was tested in two guinea pigs. In the sera taken four weeks after the first immunization, the antitoxin titres were estimated with the use of the following titration points: 0.01, 0.03, 0.1, 0.3 and 1.0 IU/ml of sera. In the sera taken two weeks after the second immunization, the antitoxin content was estimated with an accuracy of ca. 0.5 IU/ml of sera. The standard toxin was a stabilized diphtheria toxin prepared in 1944 and used as stock toxin in the preparation of Schick toxin. It contains 25 Lf doses/ml, the minimum lethal dose being 0.012 ml.

Standard antitoxin was the WHO reference standard (Standard Diphtheria Antitoxin, 10 IU/ml, obtained from the State Serum Institute, Copenhagen, Denmark).

RESULTS

The antigenic potency of AP and AH prophylactics was tested four times, after 12, 27, 34 and 52 weeks' storage at 4° C.

In the first test (after 12 weeks' storage), 9 guinea pigs were immunized with each prophylactic. The immunization dose was 2×1 Lf dose in a volume of 1 ml, injected intramuscularly at a four-week interval. Two weeks after the second immunization, the animals were bled by cardiac puncture and the antitoxin level in each serum was estimated.

The results are given in Table 1.

In the second and third test, the immunization dose was again 2×1 Lf dose in a volume of 1 ml. The injections were now given

TABLE 1

DIPHTHERIA ANTITOXIN CONTENT OF SERA OF GUINEA PIGS IMMUNIZED WITH ALUMINIUM PHOSPHATE AND ALUMINIUM HYDROXIDE PROPHYLACTICS STORED 12 WEEKS AT 4°C

AP Prophylactic		AH Prophylactic	
Guinea Pig No.	IU per Ml of Serum 2 Weeks after 2nd Injection	Guinea Pig No.	IU per Ml of Serum 2 Weeks after 2nd Injection
1	2	1	1
2	4	2	4
3	6	3	3
4	8	4	2
5	2	5	2
6	2	6	3
7	9	7	3
8	0.5	8	4
9	Died	9	2
Arithmetic mean	4.2		2.7
Geometric mean	3.0		2.5

TABLE 2

DIPHTHERIA ANTITOXIN CONTENT OF SERA OF GUINEA PIGS IMMUNIZED WITH ALUMINIUM PHOSPHATE AND ALUMINIUM HYDROXIDE PROPHYLACTICS STORED 27 WEEKS AT 4°C

AP Prophylactics			AH Prophylactics		
Guinea Pig No.	IU per Ml of Serum 4 Weeks after 1st Injection	IU per Ml of Serum 2 Weeks after 2nd Injection	Guinea Pig No.	UI per Ml of Serum 4 Weeks after 1st Injection	UI per Ml of Serum 2 Weeks after 2nd Injection
1	0.03	1	1	0.01	10
2	0.03	6	2	0.3	Died
3	0.1	8	3	0.03	5
4	0.03	10	4	0.01	0.5
5	0.03	Died	5	0.03	Died
6	0.1	4	6	0.01	0.5
			7	0.03	8
			8	0.1	6
			9	0.03	8
Arithmetic mean		5.8			5.4
Geometric mean	0.05	4.5		0.03	3.4

TABLE 3

DIPHTHERIA ANTITOXIN CONTENT OF SERA OF GUINEA PIGS IMMUNINIZED WITH ALUMINIUM PHOSPHATE AND ALUMINIUM HYDROXIDE PROPHYLACTICS STORED 34 WEEKS

AP Prophylactic			AH Prophylactic		
Guinea Pig No.	IU per Ml of Serum 4 Weeks after 1st Injection	IU per Ml of Serum 2 Weeks after 2nd Injection	Guinea Pig No.	IU per Ml of Serum 4 Weeks after 1st Injection	IU per Ml of Serum 2 Weeks after 2nd Injection
1	0.3	8	1	0.1	8
2	0.1	7	2	1.0	8
3	0.3	6	3	0.3	7
4	0.1	Died	4	0.1	11
5	0.3	8	5	Died	—
6	0.1	4	6	0.001	2
7	0.3	8	7	0.3	6
8	0.3	8	8	0.01	6
9	0.3	6	9	0.3	8
10	0.3	4	10	0.3	8
11	1.0	4	11	0.3	2
12	0.3	9	12	0.01	9
Arithmetic mean		6.5			6.2
Geometric mean	0.25	6.3		0.11	6.1

subcutaneously, the interval between the injections being the usual four weeks. Immediately before the second injection, a small bleeding by cardiac puncture was made in order to obtain a picture of the antitoxin level after a single injection of the prophylactic. The results are given in Tables 2 and 3.

In the fourth test the immunization dose was reduced to 2×0.2 Lf dose in a volume of 1 ml, again given subcutaneously at a four-week interval. The antitoxin titres were estimated four weeks after the first injection and two weeks after the second. The immunization dose was reduced in order to get the possible difference in the antigenicity of the tested prophylactics more pronounced. The results are given in Table 4. In most of the guinea pigs the titres four weeks after the first immunization were below 0.01 units/ml and thus below the lowest value in our titration series.

TABLE 4

DIPHTHERIA ANTITOXIN CONTENT OF SERA OF GUINEA PIGS IMMUNIZED WITH ALUMINIUM PHOSPHAT AND ALUMINIUM HYDROXIDE PROPHYLACTICS STORED 52 WEEKS

AP Prophylactic		AH Prophylactic	
Guinea Pig No.	IU per Ml of Serum 2 Weeks after 2nd Injection	Guinea Pig No.	IU per Ml of Serum 2 Weeks after 2nd Injection
1	4	1	0.01—0.1
2	0.5	2	0.01—0.1
3	4	3	0.1
4	4	4	4.0
5	4	5	0.1
6	4	6	4.0
7	0.5	7	0.1
8	3	8	1
9	1.5	9	5
10	4	10	2
11	0.01—0.1	11	4
12	0.5		
Arithmetic mean	2.5		1.9
Geometric mean	1.4		0.5

DISCUSSION

According to the first three antigenicity tests, there seems to be little difference in the immunization power of the two investigated prophylactics. The last experiment with the reduced immunization dose possibly indicates a slight superiority of the AP prophylactic. The antitoxin titres are here more uniform and the means higher. On the other hand, it is evident — as the broad scattering of antitoxin titres shows — that the use of a very low immunization dose enhances the effect of heterogenicity of test animals on the results, and, consequently, a considerably higher number of guinea pigs should have been immunized.

In addition, it must be considered that our laboratory has a limited experience in the preparation of the AH prophylactic, whereas the production of the AP prophylactic is a routine procedure.

The results indicate also, as Holt (2) pointed out, that the antigenicity of adsorbed diphtheria toxoid seems to improve during storage.

SUMMARY

The immunizing potency of purified diphtheria toxoid adsorbed, on the one hand, on aluminium phosphate gel and, on the other hand, on the Cγ-modification of aluminium hydroxide was investigated by following the antitoxin formation in the blood of immunized guinea pigs. No clear difference in the antigenicity of these two preparation could be demonstrated. When a very low immunization dose was used, the aluminium phosphate prophylactic caused perhaps more uniform rise of the antitoxin titres and slightly higher mean values.

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FROM THE DEPARTMENT OF BACTERIOLOGY AND SEROLOGY, UNIVERSITY OF TURKU

DRUG SENSITIVITY OF TUBERCLE BACILLI

COMPARISON OF THREE DIFFERENT METHODS

by

A. E. KORTEKANGAS and ANTERO LEVANTO

(Received for publication March 5, 1954)

Many different methods have been suggested for the tubercle bacillus sensitivity test. We can distinguish three different main types: (1) a test in which bacilli are cultivated on the surface of a solid medium, with the use of some of the known tubercle bacilli media; (2) a test in a liquid medium with a cultivation of either a dispersed or a subsurface growth type; (3) a test with the slide cultivation method described by Pryce (12).

When in the spring of 1952 we began to plan a tubercle bacilli sensitivity test suited to our circumstances, we decided to take for purposes of comparison a representative example of each of the test types mentioned above. We finally chose the following three methods: (1) the Dubos solid oleic-acid-albumin method; (2) the Dubos liquid Tween 80 albumin medium method; (3) the Pryce slide cultivation method. We chose the Dubos solid oleic-acid as the medium for the first-mentioned test and not one of our more usual egg media because our earlier experience in the preparation of these egg media showed that the streptomycin is to a large extent broken down in the preparation stage, during which the medium has to be heated for some time to 80—100°C. This breakdown of streptomycin would naturally have made comparison with the other methods used much more difficult. Moreover, a more quickly readable result was to be expected with Dubos solid medium (7, 5).

As a prime object of our investigations, we set the following questions to be answered.

1. To what extent does the absolute sensitivity of tubercle bacillus strains correspond when determined by different methods?

2. Can the level of concentration that inhibits growth be easily obtained, remaining constant by each method?

3. Which method gives the quickest result, best saves labour and expensive material?

4. Can the same method be applied to a sensitivity test with different anti-tuberculous substances?

MATERIAL AND METHODS

The preparation of Dubos solid (oleic-acid-albumin) medium differed slightly from the original instructions (1), and the medium was exactly the same as that used by Frisk and Lagergren (3). The Dubos liquid (Tween 80 albumin) medium was prepared by deviating from the original instructions (1) in that the basal medium did not contain a small amount of $CaCl_2$, $ZnSO_4$ and $CuSO_4$, the omission of which Lagergren and Frisk (6) did not consider to change the growth relations, at least to any noticeable degree. The medium used in the Pryce method was prepared according to Oeding's (11) directions.

The sensitivity test techniques corresponding to each of the three different media are here abbreviated as follows: solid (=test on Dubos solid oleic-acid-albumin medium), liquid (=test in Dubos liquid Tween 80 albumin medium) and Pryce.

The dilution series of the drugs under investigation were prepared so that the basic solution common to all methods was diluted either with saline (pro solid) or with the medium itself. Thus, the theoretical concentrations of the drug in the plates and tubes under investigation were 50.0, 12.5, 3.1, 0.8, 0.2 and 0.05 γ/cc. The highest concentration used with isoniazid (INH) was 12.5 and the lowest 0.01. For practical reasons, the test with the Pryce method was made with only the five lowest concentrations.

In our experiments we used only fresh dilution series which were prepared immediately before inoculation.

The strains of tubercle bacilli investigated were obtained from routine laboratory specimens. They were cultivated from recently grown colonies on either Dubos solid or on Löwenstein medium (Finlayson modification) — as many colonies as possible were taken — in the Dubos liquid medium

in which the strain was growing for 5 to 15 days, or until the medium had become a little diffusely turbid when the sediment on the bottom was shaken. Then the cultures for the dilution series were made. A loopful of shaken strain culture was cultivated on the solid method plate. Similarly, each liquid method tube was inoculated by a loopful of the strain culture, and for the Pryce method a loopful was smeared on the slide so that the culture area extended above the split surface of the medium when the slide was immersed in it.

In the opening stages of the experiment, the drugs investigated were dihydrostreptomycin (Merck), aminosalyl (Na-PAS Ferrosan) and Conteben (Bayer). At this stage of our work we did not obtain any useful results by the Pryce technique. Later, it became apparent that the reason for this number of failures was a technical one. If the smear placed on the slide was allowed to become very dry, the beginning of the growth was very irregular, and very often, despite prolonged incubation time, no growth could be ascertained even on the control slides. In the later stages of experiment with the Pryce method, we allowed the inoculated slides to become only just dry at room temperature, and as soon as the liquid sheen had disappeared from the area of cultivation, the slides were put into their tubes to be incubated. By proceeding in this way, we also managed to obtain reliable readings with Pryce method.

The readings with the solid and liquid methods were made on the 5th, 7th, 9th, 12th, 15th, 25th and 30th days. On the final reading, any atypical colonies were verified by staining according to Kinyoun (4). With the liquid method, it was verified by blood ágar culture and by staining of bacilli that only the tubercle bacilli had caused the turbidity in all the turbid tubes.

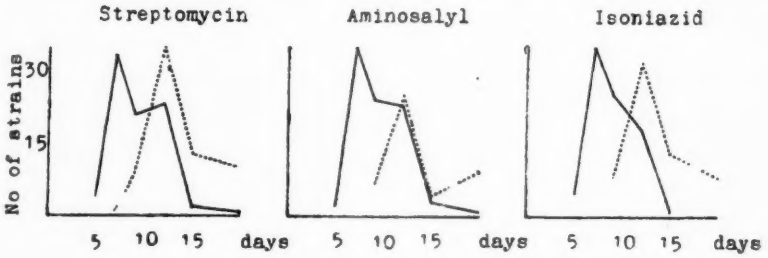
In the Pryce method we performed control slide stainings according to Kinyoun beginning with an incubation period of 7 days, and it was ascertained that generally 10 days was the most advantageous incubation period.

Later, when INH could be obtained, we dropped the Conteben sensitivity test and instead used INH. For the above-mentioned reason, Conteben sensitivities could not be proved reliably by the Pryce technique. However, in Table 4 comparison of the results on the solid and liquid methods is shown with Conteben. The initial failure of the Pryce technique also had the result that throughout the tests the solid-liquid comparison could be made with a considerably greater number of strains. In the liquid method particularly, contaminations also caused variations in the number of strains compared with the results in other methods.

Thus, all three (two) methods under comparison were always performed with every strain at the same time and from the same strain culture.

RESULTS

The comparison of our methods is arranged so that the inhibitory concentrations obtained on two methods are compared with each other. Two readings are presented with the solid and liquid methods



Figs. 1—3. — Diagrams Showing Times of Primary Readings.
——— =solid method ····· =liquid method

— the so-called primary result and the final result. With Pryce's method, naturally only one result can be shown. The »final result» was always read after an incubation of 30 days. The »primary result» was the earliest result considered worth reading (with reference to the control).

Figs. 1 to 3 show the incubation time needed for the primary result in those strains for which a comparison with all three methods could be made. The diagrams show that with the solid method the primary result could most generally be read after an incubation period of 5 to 12 days. On the other hand, with the liquid method the primary result could often be obtained only after 20 days. Different medium »sets» were seen to have (in relation to the control strain) small differences in the speed of growth, of more than two days in exceptional cases only. The individual differences in the speed of growth of the strains were noticeably greater than the differences due to the different medium »sets».

In Tables 1 to 4 the possible ratios of the inhibitory concentrations are shown as abscissae. For example, 1 indicates that the methods compared have the same inhibitory concentrations; ¼ means that in the pairs compared the inhibitory concentration of the method given as the numerator is only one-quarter that of the method shown as the denominator, and so on. In the ordinate are listed the six method pairs under comparison. We have compared the figures of both the solid and the liquid as well after primary as final readings with the Pryce, so that the amount of increase in the inhibitory concentrations between the primary and the final result appears in the table.

It appears from the tables that the inhibitory concentrations

TABLE 1

STREPTOMYCIN

Comparison Pairs	Number of Strains Compared	Ratios of Inhibitory Concentrations										
		1/1024	1/256	1/64	1/16	1/4	1	4	16	64	256	1024
1) Ic. liquid (prim.) / Ic. solid (prim.)	171				5	23	128	15				
					3 %	13 %	75 %	9 %				
2) Ic. liquid (final) / Ic. solid (final)	177			1	10	40	101	22	1	1	1	
				½ %	6 %	23 %	57 %	12 %	½ %	½ %	½ %	
3) Ic. Pryce / Ic. solid (prim.)	96					3	12	33	35	11	2	
						3 %	13 %	34 %	36 %	12 %	2 %	
4) Ic. Pryce / Ic, solid (final)	96				2	6	31	40	12	4	1	
					2 %	6 %	32 %	42 %	13 %	4 %	1 %	
5) Ic. Pryce / Ic. liquid (prim.)	54						5	10	24	12	1	2
							9 %	18 %	45 %	22 %	2 %	4 %
6) Ic. Pryce / Ic. liquid (final)	60					1	15	21	16	6	1	
						2 %	25 %	35 %	26 %	10 %	2 %	

In Tables 1 to 4, I c. means inhibitory concentration of the drug in the method in question. In the ratio of each inhibitory concentration is the number of strains and the percentage division which corresponds to it. See text for explanation of the tables.

TABLE 2

AMINOSALYL

Comparison Pairs	Number of Strains Compared	Ratios of Inhibitory Concentrations										
		1/1024	1/256	1/64	1/16	1/4	1	4	16	64	256	1024
1) Ic. liquid (prim.) / Ic. solid (prim.)	125				5	38	73	9				
					4 %	30 %	59 %	7 %				
2) Ic. liquid (final) / Ic. solid (final)	130			10	24	42	45	8	1			
				8 %	18 %	32 %	35 %	6 %	1 %			
3) Ic. Pryce / Ic. solid (prim.)	99	1			1	4	17	28	37	9	1	1
		1 %			1 %	4 %	17 %	28 %	38 %	9 %	1 %	1 %
4) Ic. Pryce / Ic. solid (final)	99	1		2	3	22	32	22	16	1		
		1 %		2 %	3 %	22 %	33 %	22 %	16 %	1 %		
5) Ic. Pryce / Ic. liquid (prim.)	40						2	11	15	11	1	
							5 %	27 %	38 %	27 %	3 %	
6) Ic. Pryce / Ic. liquid (final)	45				1	1	8	18	12	4	1	
					2 %	2 %	18 %	40 %	27 %	9 %	2 %	

TABLE 3

ISONIAZID (INH)

	Comparison Pairs	Number of Strains Compared	Ratios of Inhibitory Concentrations										
			1/1024	1/256	1/64	1/16	1/4	1	4	16	64	256	1024
1)	Ic. liquid (prim.)	74					18	52	4				
	Ic. solid (prim.)						25 %	70 %	5 %				
2)	Ic. liquid (final)	76					16	30	23	7			
	Ic. solid (final)						21 %	40 %	30 %	9 %			
3)	Ic. Pryce	96				1	13	53	25	4			
	Ic. solid (prim.)					1 %	14 %	55 %	26 %	4 %			
4)	Ic. Pryce	96				12	33	37	12	2			
	Ic. solid (final)					12 %	35 %	39 %	12 %	2 %			
5)	Ic. Pryce	53					7	29	14	2			1
	Ic. liquid (prim.)						13 %	55 %	26 %	4 %			2 %
6)	Ic. Pryce	55			3	11	21	14	6				
	Ic. liquid (final)				6 %	20 %	37 %	26 %	11 %				

TABLE 4

CONTEBEN

	Comparison Pairs	Number of Strains Compared	Ratios of Inhibitory Concentrations										
			1/1024	1/256	1/64	1/16	1/4	1	4	16	64	256	1024
1)	Ic. liquid (prim.)	68		1	3	8	19	30	4	2	1		
	Ic. solid (prim.)			1 %	4 %	12 %	28 %	45 %	6 %	3 %	1 %		
2)	Ic. liquid (final)	67		1	5	7	11	31	9	2	1		
	Ic. solid (final)			1 %	8 %	10 %	17 %	47 %	13 %	3 %	1 %		
3)	Ic. liquid (final)	68				1	3	25	17	12	6	3	1
	Ic. solid (prim.)					1 ½ %	4 %	37 %	25 %	18 %	9 %	4 %	1 ½ %

with solid and liquid compare fairly well with each other. The inclination for the ratios in the first and second comparison pairs to remain smaller than 1 more often than greater appears everywhere except with INH (Table 3, second pair). In the third pair the inhibitory concentration with Pryce is more than fourfold but under 16-fold, however, as compared with the inhibitory concentrations obtained on solid and liquid when the primary readings are taken into account. The difference only partly evens out when

we compare the Pryce results with the other final results in which the incubation period is considerably longer. The third and fourth pairs in the different tables reveals, however, that the difference explained earlier between the Pryce and both other methods is apparent only with streptomycin and aminosalyl. With INH there are no obvious differences in the absolute inhibitory concentrations according to our methods. Further, the third and fourth pairs show that the increase of the inhibitory concentration between the primary and the final readings is on an average fourfold for all three anti-tuberculous substances, which, therefore means the difference of not even one tube in the dilution series that were used.

There appear in Tables 1 to 4 only some discrepancies in the streptomycin sensitivity tests performed by different methods, and nearly all of these could be explained from the statistical point of view as being caused by biological deviation. The discrepancy in the results is greater with aminosalyl than with streptomycin, and quite obvious discrepancies appear oftener. With INH there is only one clear discrepancy between the liquid (primary) and the Pryce results, and this is due only to the slow growth and is eliminated when the liquid (final) reading is taken into account.

Sometimes contamination spoiled the sensitivity test. With the regular comparison material of 100 strains, the result of the solid and the Pryce was not once disturbed by contamination. On the other hand, because of contamination the liquid result had to be scrapped 6 times with streptomycin, 19 times with aminosalyl and 8 times with INH.

With the Pryce, reading the results takes more time than with the liquid and especially with the solid method. Costs are considerably greater for solid and liquid media than for the Pryce medium. This is due to the high price of bovine plasma fraction V. Our experiments with the corresponding human plasma fraction showed that it was less suitable.

All the methods used by us have proved to be of equal value. A comparison of the inhibitory concentrations obtained in tests of the control strain (H37Rv) may give the best picture of this (Table 5).

TABLE 5

INHIBITORY CONCENTRATIONS (γ/ML) OF THE CONTROL STRAIN (H37RV)

Method	Streptomycin			Aminosalyl			Isoniazid (INH)			Conteben		
	Number of Tests	Usual Variation	Occasional Variation	Number of Tests	Usual Variation	Occasional Variation	Number of Tests	Usual Variation	Occasional Variation	Number of Tests	Usual Variation	Occasional Variation
Solid	59			57			37			25		
Prim.		0.2—0.05	0.8 (11 times)		0.8—0.05	—		0.2—0.05	0.01 (6 times)		0.8—0.05	3.1 (once)
Final		0.8—0.2	3.1 (twice)		0.8—0.2	3.1 (8 times 0.05 (3 times)		0.2—0.05	0.8 (once) 0.01 (5 times)		50 —0.8	>50 (once)
Liquid				18			10			10		
Prim.	20	0.8—0.05	—		0.8—0.05	—		0.2—0.05	0.8 (once)		12.5—0.05	
Final		0.8—0.2	3.1 (once)		0.8—0.2	—		3.1—0.05	—		50 —3.1	>50 (once)
Pryce	8	3.1—0.8	—	8	3.1—0.8	0.2 (once)	8	0.2—0.05	—	—		

DISCUSSION

It has been demonstrated (14) that the sensitive strains of tubercle bacilli, even when not susceptible to the influence of streptomycin, form on growing less sensitive variants to this substance. We may suppose that the division of a similar strain into more or less resistant variants also occurs in regard to other anti-tuberculous substances (8). Thus, in the liquid medium even one viable variant would be considered as a resistant strain in the concentration in question, whereas, on the other hand, when testing the same strain on the solid medium, we could establish the small number of resistant variants compared with sensitive variants (with the growth in the control).

According to the general result of our experiment, we obtained, however, a slightly smaller inhibitory concentration with the liquid than with the solid. To this there was only one exception (Table 3, pair 2). Naturally, one explanation may be that, in the preparation stage of the solid test series, part of the anti-tubercle-bacillus substance combined with the medium or disappeared in some other way. The result can also be explained by the quality of the strains investigated, the majority of them having been isolated from untreated tuberculous patients. Further, Tween 80 which is included in our medium can produce an effect, as Mitchison (10) has shown, inhibiting the appearance of resistant variants in the liquid medium, especially if the incubation period is short and the strain relatively resistant. The last-mentioned circumstance may not really have had much effect because the greatest part of our strains represented a sensitive type and the follow-up period was 30 days. As a fourth explanation of our result, it must be remembered that only certain proportion of resistant variants is generally capable of giving rise to growth in a sensitivity test by the liquid method. According to Mitchison, in tests by the liquid method, about 3 per cent streptomycin-resistant variants are required to bring out the resistance.

As a general observation on the solid method, we may mention the following. In small concentrations a growth quicker and stronger than the control was obtained with INH. Primary readings were easily made, but in the final readings it was nearly always possible to determine 5 to 20 strongly grown colonies in the concentration

which was ¼th of the fully inhibiting concentration. This observation agrees with the view of Marshall *et al.* (8) on the division into appreciably changing variants with INH sensitivity.

Concerning the Pryce method, we can make some observations in regard to microcolonies under the effect of different anti-tuberculous substances. In the aminosalyl series, for instance, there was always a little growth to be seen, even when the strain was very aminosalyl-sensitive. Sievers (13) made similar observations about aminosalyl. The effect mechanism of aminosalyl seems to permit a small initial growth in all cases.

SUMMARY

A sensitivity test with 63 strains of tubercle bacilli was made simultaneously from the same basic cultivations (Dubos Tween 80 liquid method) by three different methods. A comparison between two methods is presented on the basis of tests made simultaneously with 157 strains of tubercle bacilli.

The absolute inhibitory concentration was the same size with all the methods investigated (solid, liquid and Pryce). A small difference was noticed between the first and last-mentioned methods in streptomycin and aminosalyl sensitivity but no difference at all in the INH sensitivity. The inhibitory concentrations obtained with some strains by different methods clearly differed from those obtained with others. Nearly all the larger differences were observed in the tests of sensitivity to aminosalyl.

All the methods proved to be sufficiently even in practice.

For routine work, we recommend either the slide method (slide cultivation test according to the method of Oeding, cheap medium) or the solid medium test (Dubos oleic-acid-albumin). The liquid medium method is of secondary importance because of its larger sensitivity to contamination.

The same methods were successfully applied to streptomycin, aminosalyl, INH and Conteben sensitivity tests.

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FROM THE STATE SERUM INSTITUTE, HELSINKI, AND THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

THE NATURE OF COLI HEMOLYSINS

by

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Coli hemolysins are labile substances and difficult to produce. Some experiments seem to indicate that only living E. coli bacteria can hemolyze red cells. The hemolytic activity of a coli culture in Robinson's synthetic medium thus vanishes when the bacteria are removed. Widholm (1953) suggests the existence of true extracellular coli hemolysins to be more probable. Robinson (1953), however, is not of the same opinion. A further study of this matter was thus desirable.

EXPERIMENT

The hemolytic activity of the solutions to be tested was quantitatively measured in serological exponential dilution series in a water-bath at 37° C for 1 hour. A 2 per cent suspension of sheep red cells was used as an indicator (2).

In order to examine the effect of growing coli bacteria on the activity of coli hemolysin, 1 mg of streptomycin was added per ml of an incubated hemolytic coli culture in 1 per cent peptone water medium. The activity of the solution was unchanged after the addition, giving complete hemolysis at dilution 1/64. Storage for one hour at 4° C and centrifugation in a Spinco centrifuge (diameter of rotor 26 cm) at the rate of 10 000 r.p.m. for 30 min. did not alter the activity. Cultures of the centrifuged solution on blood agar plates showed no bacterial growth after 24 hours at 37° C. The original culture and the centrifuged solution had an activity of

1/8 and 1/32, respectively, after 18 hours at 4° C. A parallel control test with plain peptone water and streptomycin showed no activity.

On the basis of similar results obtained in repeated series the purification of coli hemolysin was continued. In addition to the methyl alcohol precipitation used by Widholm (2), the precipitation with zinc-ion was studied. *E.g.*, 370 ml of active coli culture in 1 per cent peptone water was centrifuged in the Spinco centrifuge at the rate of 10 000 r.p.m. for 30 min. The activity of the solution was 1/64 after centrifugation. The solution was brought to 0.01 M zinc by the addition of 7.4 ml of 0.5 M zinc-acetate. The addition was made with stirring at 0° C, *p*H 6.6. After centrifugation at 0°C, 3000 r.p.m., for 30 min. the precipitate was mixed with 50 ml of 0.9 per cent NaCl which contained 2 mg of streptomycin per ml. The suspension was thereafter dialyzed against 2 liters of acetate buffer of *p*H 6.0, ionic strength 0.15, at 4° C for 18 hours with continuous stirring. The dialysate was then centrifuged at 0° C, 3000 r.p.m., for 20 min. The supernatant showed an activity of 1/256. Compared with the original volume (370 ml) the supernatant (50 ml) contained ca. 50 per cent of the starting activity. Cultures of the solution on blood agar plates showed no bacterial growth after 24 hours at 37° C. Parallel preparations from plain peptone water for control gave a solution with no hemolytic activity.

By performing similar precipitations from coli cultures to which streptomycin had been added before the Spinco centrifugation hemolytic solutions were repeatedly obtained. The hemolytic activity of the solutions could be stored by means of freeze-drying.

SUMMARY

The preparation of a powder showing hemolytic activity is described. The isolation was done from a peptone water coli culture containing streptomycin using zinc-ion precipitation, dialysis and freeze-drying.

The hemolytic activity of coli hemolysin solutions containing streptomycin suggested that the effect of coli hemolysin is not necessarily connected with the simultaneous growth of coli bacteria.

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FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI, AND FROM THE UNIVERSITY DEPARTMENT OF THE TUBERCULOSIS HOSPITAL, HELSINKI

EXPERIMENTAL STUDIES OF THYROID GLAND REACTION DURING TREATMENT WITH PARA-AMINOSALICYLIC ACID, DIHYDROSTREPTOMYCIN, ISONIAZID AND VIOSIN

by

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Increasing attention is being paid in the literature to the relationship between tuberculosis and the thyroid gland. This has been made necessary not only by the findings in recent endocrinological studies but particularly by the observation that the drugs used in the treatment of tuberculosis influence the function of certain endocrinological organs. So far it is best known that para-aminosalicylic acid (PAS), when used over a long period, may give rise to goitre or transient myxoedema (2, 5, 6). Experimental studies, also, have confirmed the action of PAS on the thyroid. Kjerulf-Jensen and Wolffbrandt (1951) found cellular hyperplasia in rats after PAS administration and Wong *et al.* (1953) observed a reduced I^{131} uptake but no effect on the pituitary-adrenal axle after 2 weeks' administration of PAS to rats. Beattie and Chambers (1953) studied changes in the thyroid as a function of the length of PAS therapy and found a reduced radio-active iodine uptake in rats following a brief treatment period of 16 days. When administration was continued for about 4 months (15 weeks) the values were more than twice as high as those of the control series.

It was the purpose of this investigation to study the effect on the thyroid gland not only of PAS but also of dihydrostreptomycin, isoniazid and Viocin, using as criterion the linear measurement

method of quantitative histologic determination of thyroid activity developed by Uotila and Kannas (1952). Changes in the weight of the thyroid, thymus, liver, spleen, kidneys, adrenals and testes after treatment were also studied.

MATERIAL AND METHODS

The experimental series consisted of 80 adult male guinea pigs of 450—650 gm body weight. The daily dosages and manner of administration of the different drugs was as follows: PAS, 100 mg *per os* (through gastric tube); isoniazid (Rimifon) 10 mg *per os* (gastric tube); dihydrostreptomycin, 6 mg injected subcutaneously; Viocin (Pfizer), 10 mg by subcutaneous injection. The daily dosages corresponded to the therapeutic doses for guinea pig tuberculosis mentioned in the literature. There were also two control series, one of which received 1 ml of tapwater *per os* and the other 1 ml of physiological saline solution by injection. The animals were killed by decapitation and the thyroid gland was dissected out, weighed on a torsion balance and fixed in Bouin's fluid. The thymus, liver, spleen, kidneys, adrenals and testes were weighed and stored in formalin for histological examination. Each lobe of the thyroid was embedded in paraffin and cut longitudinally with the microtome. Several sections made from the median planes were stained by Koneff's modification of Mallory's azan method, which has proved to be best suited for the quantitative histological examination of the thyroid by the linear measurement method of Uotila and Kannas (1952). The procedure consists in the projection of the image of a stained thyroid section in a magnification of about 400 through a microprojector downward onto a white plane. The relative lengths of epithelium, colloid and stroma in each thyroid lobe of every animal was measured along straight lines drawn arbitrarily on the projected image. The length of the total distance measured was 1,600 cm per lobe and 3,200 cm per thyroid gland. The percentage of epithelium in the gland was used as indicator of thyroid activity. A comparison was made of the group averages by statistical methods.

RESULTS

Experiment 1

Two groups of 10 guinea pigs each were used in this experiment. The first group received PAS during 28 days and the second group tapwater (*per os*) for the same length of time. One animal in each group died of pneumonia on the 14th day; this apparently was aspiration pneumonia caused by the gastric tube. Table 1 shows the changes in the average body weights of the 9 animals in each group before and after treatment, the average weights, with standard errors, of the different organs, and differences between the group averages for the PAS animals and the controls.

TABLE 1

ABSOLUTE BODY AND ORGAN WEIGHTS IN PAS AND CONTROL GROUPS (GROUP AVERAGES)

	No. of Animals	Body Weight (gm)	Thyroid (mg)	Thymus (mg)	Liver (mg)	Spleen (mg)	Adrenals (mg)	Kidneys (mg)
PAS	9	576	67.0	274	24160	953	381	4853
		±33.3	±4.3	±34.9	±1570	±74	±38.6	±218
Controls	9	578	60.9	322	21450	701	299	4441
		±13.6	±3.2	±25.4	±766	±67	±17.3	±164
Difference		—2	+6.1	—48	+2710	+252	+82	+412
Confidence level		no	no	no	no	98%	94.5%	

It is noted from the table that the body weights were very similar in the two groups, for the final average body weights of 576 ± 33.3 gm and 578 ± 13.6 gm show no significant difference. The average thyroid weight in the PAS group was 67.0 ± 4.3 mg and in the control group 60.9 ± 3.2 mg, which also is not a significant difference. On the other hand, the spleen and adrenal weights show a significant increase in the group given PAS. A comparison of the relative weights of the organs gave similar results.

From table 2, which gives the results of the histoquantitative measurement of the thyroid gland, it is seen that PAS influenced the histological activity of the gland. This is evident as a statistically significant decrease in the percentage of epithelium and increase in the percentage of colloid in the gland.

TABLE 2

HISTOQUANTITATIVE MEASUREMENTS OF THE THYROID GLANDS IN PAS AND CONTROL GROUPS (AVERAGE GROUP PERCENTAGES OF EPITHELIUM, COLLOID AND STROMA)

	No. of Animals	E%	C%	S%
PAS	9	62.6 ±0.99	34.8 ±0.85	2.6 ±0.26
Control	9	68.0 ±1.25	29.1 ±1.24	2.9 ±0.19
Difference		—5.4	+5.7	—0.3
Confidence level		99.6%	99.9%	

Experiment 2

For this experiment 6 groups of 10 guinea pigs each were employed. The drugs were administered for a period of 8 weeks, or for a considerably longer time than in the preceding experiment. The first group consisted of control animals, which were given tap-water *per os*. The second group received PAS as in the preceding test and the third Rimifon. The fourth group was again a control and was given injections of physiological saline. The fifth group received streptomycin and the sixth Viocin. In the course of the test 3 animals died in the first control group, 2 in the second control group, and 2 in the PAS group. In most cases the cause of death was pneumonia. Decapitation of the remaining 53 animals, preparation of the histological specimens and examination of the results were carried out as in Experiment 1. The average body and organ weights in the different groups and their comparison with the control group averages are given in table 3. Table 4 shows the results of the histoquantitative measurement of the thyroid gland.

As will be observed, none of the drugs used produced statistically significant changes in the average thyroid weight in the different groups at the end of an 8 weeks' administration. As regards the weights of the other organs examined, the average thumus weight increased significantly in the Rimifon group and decreased in the dihydrostreptomycin group. The weight of the liver was higher in the animals receiving dihydrostreptomycin and that of the spleen was reduced in the animals which were given Rimifon. The adrenals increased in weight in the PAS as well as in the Viocin group. The

TABLE 3

ABSOLUTE BODY AND ORGAN WEIGHTS IN PAS, RIMIFON, DIHYDROSTREPTOMYCIN, VIOCIN AND CONTROL GROUPS (GROUP AVERAGES)

	No. of Animals	Body Weight (gm)	Thyroid (mg)	Thymus (mg)	Liver (mg)	Spleen (mg)	Adrenals (mg)	Kidneys (mg)	Testes (mg)
Controls (per os)	7	527	77.0	266	22186	922	317	3929	3265
		±37.6	±6.6	±44.9	±1014	±62.7	±24.1	±199	±434
PAS	8	554	86	281	22219	879	426	4013	3412
		±18	±4.3	±13.5	±417	±234	±41.8	±90	±380
Difference		+27	+9	+15	+33	—43	+109	+84	+147
Confidence level							95%		
Rimifon	10	545	75	409	21620	593	290	3638	3680
		±20.2	±2.9	±19.8	±760	±29.3	±15.9	±104	±126
Difference		+18	—2	+143	—566	—329	—27	—291	+415
Confidence level				99.2%		99.1%			
Controls (inject.)	8	634	79	419	27200	603	402	3870	3980
		±21	±4.5	±44.6	±1100	±72.5	±23.1	±104	±183
Dihydrostrepto-mycin	10	542	77	301	21060	577	336	3819	3692
		±26.2	±3.7	±33.9	±968	±53.0	±23.5	±137	±212
Difference		—92	—2	—118	—6140	—26	—66	—51	—288
Confidence level				96%	99.2%				
Viocin	10	613	78	366	26660	746	622	4780	4333
		±29.6	±9.6	±29.2	±964	±38.1	±98.5	±137	±209
Difference		—21	—1	—53	—540	+143	+220	+910	+353
Confidence level							95%	99.9%	

latter also had heavier kidneys. No significant differences were seen in the average group weights of the testes. Since the test animals were not of identical body weight in the different groups, the results were also compared on the basis of the relative weights of the organs. This comparison gave values fairly similar to those obtained in the comparison of the absolute weights. The only deviation from the latter results was a decrease of the significant difference between the PAS and control group adrenal weights.

According to the measurements of histological activity of the thyroid gland, the results of which are shown in table 4, there was a significant decrease (with 95.5 per cent confidence) in the average proportion of epithelium in the thyroids of animals in the Rimifon group (E% —6.6) as compared with the corresponding control group. A similarly significant increase in the colloid percentage

TABLE 4

HISTOQUANTITATIVE MEASUREMENTS OF THE THYROID GLANDS IN PAS, RIMIFON DIHYDROSTREPTOMYCIN, VIOCIN AND CONTROL GROUPS (AVERAGE GROUP PERCENTAGES OF EPITHELIUM, COLLOID AND STROMA)

	No. of Animals	E%	C%	S%
Controls (per os)	7	63.8±1.70	33.7±1.63	2.5±0.23
PAS	8	67.7±2.25	29.6±2.3	2.7±0.33
Difference		+3.9	—4.1	+0.2
Confidence level		no	no	no
Rimifon	10	57.2±1.85	40.8±2.07	2.0±0.28
Difference		—6.6	+7.1	—0.5
Confidence level		95.5%	no	no
Controls (injection) ..	8	68.2±2.02	28.8±1.87	3.0±0.43
Dihydrostreptomycin ..	10	64.1±2.23	33.7±2.29	2.2±0.20
Difference		—4.1	+4.9	—0.8
Confidence level		no	no	no
Viocin	10	63.7±1.68	33.9±1.60	2.4±0.25
Difference		—4.5	+5.1	—0.6
Confidence level		no	95.5%	no

was seen in the Viocin group. The dihydrostreptomycin group also showed a decrease in the percentage of epithelium and increase in the percentage of colloid, but these difference were not statistically significant.

DISCUSSION

The results of these animal experiments point to the possibility that, of the tuberculostatic drugs tested by us, especially Rimifon and Viocin, and to some extent PAS, have an inactivating effect on thyroid function when the percentages of different histological components of the thyroid gland are used as indicator. On the other hand, dihydrostreptomycin, although it appeared to have a similar action, did not cause a statistically significant effect. These findings are compatible with the results obtained earlier in clinical experience and animal experiments with para-aminosalicylic acid (1, 8). It further appears probable on the basis of our results that after administration of the drug for a longer time the inactivating action of para-aminosalicylic acid on the thyroid gland is lost or becomes an activating

one, as demon- strated by Beattie and Chambers, who used as indicator the uptake of radio-active iodine by the thyroid gland of rats.

In our experiments the average group weight of the adrenals of the animals receiving PAS showed definite increase after administration for 4 or 8 weeks. This speaks in favour of the opinion that the effect of PAS on the thyroid is to be regarded chiefly as a part of the general adaptation syndrome, in the same manner as Cronheim *et al.* (1953) have interpreted the effect of salicylic acid on the adrenal-pituitary system. Our results differ in this respect from those of Wong *et al.* (1953), who found no signs of changes in the adrenal function of the rat after 2 weeks' administration of PAS. It is to be kept in mind, however, that the phenomena here studied usually change closely as a function of time. Whether or not the results obtained are interpreted on the basis of the general adaptation syndrome, they nevertheless appear to indicate that endocrinological factors may play an important part in the use of these drugs in the treatment of tuberculosis.

SUMMARY

1. Para-aminosalicylic acid (PAS) administered for 4 weeks caused a significant decrease in the percentage of epithelium in the guinea pig thyroid as compared with the control group. Viocin and isoniazid (Rimifon) produced a similar effect after 8 weeks' administration, whereas no change in the proportions of the histological components of the thyroid gland was observed following the application of PAS during 8 weeks.

2. Dihydrostreptomycin was not found to have a significant effect on the histoquantitative relations in the thyroid, although the result seen appears to point in the direction of effects similar to those in the preceding groups.

3. The adrenal weights were increased in the PAS and Viocin groups. In association with the histoquantitative changes in the thyroid gland, this is probably due chiefly to the effect of these drugs on the adrenal-pituitary system.

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A WEAK B CONTAINING ANTI-B

by

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Shortly after the discovery of the divisibility of the A blood group into two subgroups attempts began to be made aiming at a similar division of the B group. These efforts have been less fruitful than those concerned with the A group. No »anti-B_1» has been produced by absorbing anti-B sera, and as far as we know only one distinct anti-B has been found in a person with the agglutinogen B. It has been reported by Formaggio (2) and was discovered in an AB adult. The optimum temperature for agglutination was 8°—12°. No further data about the serum or about the antigen of the donor has been given.

In default of »anti-B_1» serum the endeavours to divide the B group have been, in the main, quantitative examinations of the agglutinability and absorptive power. The researches carried out by Worsaae led him to conclude that the B group, in contrast with the A group, forms a unity (12). In 1937, Matta proposed two differentiable groups B_1 and B_2 though less distinct than A_1 and A_2 (8). Wiener, in 1943, considered that, in spite of differences within the agglutinogen B, at the then stage of research they were of no practical importance (10). The studies in the agglutinability of B cells by anti-O sera conducted by Hirszfeld in 1947 gave 14.8% as the frequency of B_2 in Warsaw. He made a further division of B_1 into the pleiads B_m, B_r and B_j (4). Formaggio determined the agglutinability and absorptive power of 250 samples quantitatively. He used several different anti-B and anti-O sera; in addition, he had

the above irregular human anti-B at his disposal. He noticed that the differences within the agglutinogen B were quantitative rather than qualitative (2).

We have discovered a blood specimen, the cells of which are agglutinated by anti-B sera. Its serum agglutinates A and AB cells strongly, B cells to a less extent and O cells not at all. The specimen was found in Mr. O. Vuorio, aged 51, who had never been given transfusions and who was not a twin. Otherwise his blood group was MN, C+ D+ E+ c+, K—, Le (a—b+). The direct anti-human globulin test was negative. The results from closer examinations were as follows.

THE AGGLUTINABILITY OF CELLS

The cells of O.V. were tested against 32 A sera, 38 B sera, 12 O sera, and 4 AB sera. They were agglutinated by all O sera, all A sera except two, but they were not agglutinated by any of the B or AB sera. Most of the sera that caused an agglutination were titrated. The titres though lower than those obtained with the B cells were rather parallel. Table 1.

TABLE 1

THE AGGLUTINATION OF THE CELLS OF O. V. BY VARIOUS HUMAN NORMAL SERA

Kind of Serum	Number of Sera Agglutinating the Cells in the Dilution of						
	No aggl.	1/1	1/2	1/4	1/8	1/16	1/32
A	2	6	2	6	2	4	1
B	38	—	—	—	—	—	—
O	—	1	2	2	4	1	—
AB	4	—	—	—	—	—	—

The agglutination was inhibited by B and AB cells and saliva from B and AB secretors. Neither A nor O cells nor the saliva of A or O secretors or of non-secretors inhibited it.

The agglutinability of the cells of O.V. by the agglutinins of *Cytisus sessilifolius*, *Marasmius oreades* and *Sophora japonica* was also tested. They were agglutinated by the anti-O (H) extract from *Cytisus sessilifolius* (9) like the weakest O cells. When agglutinated by the extracts of *Marasmius oreades* (1) and *Sophora japonica* (5)

they yielded titres that were higher than the titres of the O cells by one or two powers of 2. They lagged, however, considerably behind the B cells.

ABSORPTION AND ELUTION

The cells of O.V. absorbed anti-B from normal human sera very weakly. When the proportion of the cells and sera was $^1/_4$ to $^1/_2$, the titre of the serum fell by approximately one power of two. The proportion of $^1/_1$ gave the results shown in Table 2.

The elution experiments were carried out according to the technique of Landsteiner and Miller (6). Anti-B could be easily eluted from the treated cells of O.V. When the cells were incubated in O-serum, a small quantity of anti-A could be demonstrated in

TABLE 2

TITRES AGAINST B AND O.V. CELLS OF A GROUP A SERUM BEFORE AND AFTER ABSORPTION WITH VARIOUS KINDS OF CELLS IN THE PROPORTION OF 1/1

		Titres against Cells	
		B	O.V.
Unabsorbed		64	4
Absorbed with	A_1	64	4
	A_2	64	4
	B	—	—
	O	64	4
	A_1B	2	0
	A_2B	—	—
	O.V.	16	1

TABLE 3

TITRES AGAINST A, B AND O. V. CELLS OF DIFFERENT ELUATES

Eluates from	Titre against Cells		
	B	O. V.	A
A_2 cells treated with A serum	—	—	
A_1 » » » » »	—	—	
B » » » » »	8	—	
O » » » » »	—	—	
A_1B » » » » »	128	—	
A_2B » » » » »	16	—	
O.V. » » » » »	32	16	
O.V. » » » O »	16	8	2
B » » » » »	16	—	2

the eluate. (Table 3). It is to be noted that the agglutinin obtained in this way was almost as strong against the cells of O.V. as against usual B cells in contrast with the ordinary anti-B sera and the eluate obtained from AB cells. Anti-A could not be eluted from the cells of O.V. treated with anti-A serum.

IMMUNISATION OF HUMAN VOLUNTEERS

Two volunteers belonging to the O group were given 1 ccm of the blood of O.V. intravenously. Samples were taken before as well as 3 and 10 days after the injection. In both cases the anti-B titre had risen by one power of two while the anti-A titre had remained the same.

SALIVA

Mr. O. V. was found to be a secretor of Le^b and H substances. It was interesting to see if he could be shown to be a secretor of B substance, too. In order to avoid unspecific inhibition, the saliva was titrated from the dilution of $^1/_4$ on. In that dilution it did not at all inhibit the agglutination of A cells by anti-A. It did inhibit agglutination by anti-B weakly when normal B cells were used as test cells and more strongly when the test cells were from his own blood (Table 4). Grove-Rasmussen *et al.* have found similar characteristics in the saliva of one weak A (3).

TABLE 4

THE DILUTIONS OF THE SALIVA OF O.V. WHICH INHIBIT ANTI-A AND ANTI-B FROM AGGLUTINATING A, B AND O.V. CELLS

	Test Cells		
	A	B	O.V.
Anti-A	No inhibition		
Anti-B		16	64

SERUM

The serum of O.V. agglutinated various kinds of red cells as shown by Table 5. Thus it seems that the serum contains anti-A and anti-B like normal O-serum. The quantitative differences between these two agglutinins, however, are far greater than usual.

TABLE 5

TITRES OF THE SERUM OF O.V. AGAINST VARIOUS KINDS OF RED CELLS

	Number of Cell Samples Being Agglutinated by the Serum in the Dilution of							
	No Aggl.	1/1	1/2	1/4	1/8	1/16	1/32	1/64
A	—	—	—	—	—	7	5	1
B	—	2	13	27	1	—	—	—
O	35	—	—	—	—	—	—	—

The serum of O.V. did not agglutinate B cells at 37° C. At 4°C the reaction was slightly stronger than at room temperature. It did not agglutinate his own cells at any temperature.

The titres of the serum when checked against different B cells showed rather parallel variability with ordinary anti-B.

Human B cells absorbed the anti-B of O.V., and it could be eluted from them. No anti-B could be eluted from the cells of O.V. whether they had been treated with a quantity of his own serum or left totally untreated.

The agglutination of B cells caused by the serum of O.V. was not inhibited by his own saliva. Likewise saliva rich in human B substance inhibited the agglutination only weakly.

RELATIVES

Unfortunately, Mr. O.V. was childless and his parents were dead. The total number of his brothers and sisters, who were alive was 6. Of these we were able to find 5. They all belonged to the O blood group and their serum agglutinated the cells of O.V.

DISCUSSION

There seem to be three questions to discuss.

A) Do the cells of O.V. possess the B characteristic?

B) Is the possible B characteristic different, qualitatively, from the normal B or is it similar to it though very weak?

C) Does the anti-B which is to be found in the serum of O.V. represent the normal even if weak anti-B or an anti-B with a missing component, which supposed anti-B might be assumed to divide the B group into two categories?

A) The following are arguments against O. V.'s belonging to group B.

1) Supposing the group were B, O. V. would represent a type which, in all probability, would be exceedingly rare.

2) None of the brothers and sisters examined had this characteristic peculiar to O. V., though it could have been easily demonstrated. If either of his parents had had it dominant, there is a 9.4% probability of its being inherited by one, and only one, of 6 children.

A discovery recently reported by v. Loghem and v. d. Hart (7) adds considerable interest to this problem. They had found a family consisting of group O parents with 3 A_4 children. Besides illegitimacy they suggested two possible explanations to this mystery. One was the recessivity of the determinator of these weak A_4's. In this case the gene should have been single in both parents. The other explanation was an epistasis by some blood group. The only possible factor would have been Le^b; the A_4 children were all Le (a+b—).

In our case the genetical variance is only a matter of probability. The possibility of illegitimacy is indisputable. An epistasis by Le^b of the weak B is no explanation because the propositus is Le (a—b+).

Supposing the gene determining the unusual B characteristic were recessive being single in both parents, $^3/_4$ of the children would belong to the blood group O. This would agree perfectly with the distribution 5 to 1.

All the results from the experiments described above support, more or less strongly, the assumption that O. V.'s cells possess the B characteristic. The results have been read blindly for the most part. The arguments against the theory are statistical only. Therefore, we believe, Mr. O. V. must be regarded as a weak B.

B—C) It is generally believed that a serum cannot permanently contain antibodies that counteract a person's own antigens. This seems to be true in the present case, too, because from the cells of O. V. treated with his own serum agglutinins cannot be eluted at all, nor are they agglutinated by anti-human globulin serum.

The cells of O. V. seem to differ from normal B cells also qualitatively as it has been possible to prepare such eluates from

anti-B sera as agglutinate both kinds of these cells identically, while other eluates prepared from the same sera, though agglutinating B cells in the dilution of 1/128, do not agglutinate O. V.'s cells at all. See Table 3.

The cells of O. V. do not seem to belong to Wiener's hypothetical group B' (11) because, first, after an incubation in O serum a small quantity of anti-A or anti-C can be eluted from them; secondly, because they are, though weakly, agglutinated by the eluate which is obtained from A cells treated by O serum. According to Wiener this eluate would contain only anti-A and anti-C, not anti-B.

The anti-B of O. V. seems to differ from the ordinary anti-B only in that it is unusually weak and fails to agglutinate the cells of O. V., unlike some equally weak anti-B sera. It may be mentioned as an interesting point that the saliva of B secretors inhibits an agglutination by the anti-B of O. V. only very slightly.

We are of the opinion that the cells of O. V. differ greatly from normal B cells; and that his serum contains an anti-B rather similar to the normal anti-B only deficient in those components which counteract his own cells. A further study concerning the matter is under preparation.

SUMMARY

A blood specimen is described, the cells of which were agglutinated in anti-B sera, and whose serum contained anti-A and anti-B. The cells responded to agglutination, absorption, elution and immunisation tests like very weak B cells, differing however from them qualitatively, too. The saliva contained B substance in minute quantities. The anti-B of the serum was weak, it left its own cells unagglutinated.

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FROM THE SECOND MEDICAL CLINIC AND THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

OCCURRENCE OF YEAST-LIKE FUNGI IN SALIVARY FLORA AND THE EFFECT OF ANTIBIOTIC THERAPY

by

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INTRODUCTION

Increasing attention is being paid by clinicians and investigators to the possible untoward effects of antibiotic therapy (2, 3, 6, 14, 27). One of the undesirable effects is the disturbance in the ecologic balance which occurs when antibiotics destroy the autochthonous bacterial flora. A number of cases have been reported in which new bacterial infections have developed during treatment with antibiotics (1, 8, 11, 13, 15, 18, 21, 22). A very dangerous circumstance is the marked increase in monilias and other yeast-like fungi during wide spectric antibiotic treatment, resulting in fatal pulmonary moniliasis especially in patients with previous chronic pulmonary disease, such as abscess of the lung or bronchiectasis (6, 16, 20, 23, 30, 32). Autopsies, also, have revealed generalised changes produced by yeast fungi after lengthy penicillin therapy (7, 26).

Attempts have also been made to obtain experimental evidence on this subject. Campbell and Saslow (4) observed that streptomycin increases the growth of certain species of fungi in vitro but were unable to find a similar effect in vitro. Foley and Winter (9) found that *Candida albicans* killed chicken embryos more readily in the presence of penicillin; however, they could not accelerate the growth in vitro. In the experiments of Lipnik *et al.* (19), the growth of Candida albicans was not stimulated in vitro by aureomycin, chloromycetin, terramycin or penicillin. These workers reached the

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same result in experiments with mouse moniliasis but the conclusions drawn were not as definite. The highly doubtful results obtained experimentally seem to be evidence that antibiotics do not exert a direct stimulating action on the growth of yeast-like fungi.

Affections of the mucosa of the mouth and the intestines have been encountered in association with antibiotic therapy. Such changes have been found in as many as 6—20 per cent of cases treated with aureomycin or chloromycetin (1, 2), and they have also been observed during treatment with penicillin and even with sulfa drugs. Various explanations have been offered for this complication and the inference has been made that it is due to the prominence of moniliasis produced by Candida albicans. Although this yeast-like fungus is very commonly present in these cases, Tomaszewski (29) is of the opinion that the occurrence of the fungus is purely incidental and that it has no definite etiological importance. The simultaneous occurrence of thrush, monilias, and disturbance in the vitamin B metabolism during antibiotic therapy still remains unexplained etiologically (12, 17, 29). There is apparently a combined causal connection and some further factor probably exists also.

It is fairly difficult to definitely ascribe an infection to fungi. Yeast-like fungi are fairly common parasites in the human organism as well as elsewhere in nature (28). In Finland they are normally encountered in the mouth flora of about 30 per cent of healthy persons (31). Woods *et al.* (32) stress that before conclusions can be drawn regarding changes in the fungus flora, the incidence of fungi among the local population must be well known. Since antibiotic therapy is very commonly used in medical hospitals, the authors therefore considered it desirable to make a *survey of the fungus status of the patients* in one of these hospitals and to see at the same time whether *changes in the fungus flora during the usual antibiotic treatment* can be determined by the available routine methods.

PRESENT INVESTIGATION

Material and Methods. — Our series consisted of 292 adults of varying age who had been admitted to our hospital for examination and treatment because of some diagnosed or suspected disease. The material for this study was collected during a period of 6

months and consisted of saliva samples from each patient. During the first 3—5 days after admittance a sample was taken daily in the morning before the ingestion of food or drink. The mouth had been cleansed on the previous evening with tap water without the use of chemicals.

A smear specimen stained with methylene blue was immediately prepared at the hospital from each sample. These specimens were examined in the University Department of Serology and Bacteriology by the same persons who examined the corresponding fungus cultures. Culture samples taken simultaneously with the direct sample were inoculated immediately on the same forenoon on three culture media (a total of 1044 cultures). The following culture media were used:

Sabouraud's ordinary fungus culture medium:
Modified *oil-Sabouraud* culture medium (10); and
Thioglycolate culture medium for anaerobic cultures (24).

When the experiments were started, an effort was made in a few cases to gradate the amount of fungi in the Sabouraud cultures by diluting the saliva in powers of 10, starting with $^1/_{10}$. Probably because of the consistency of the saliva the gradation did not give a sufficiently clear and consequent picture and its use was discontinued.

TABLE 1

FUNGUS CULTURES FROM SALIVA

Native Preparation	Ordinary Sabouraud Medium		Modified Oil-Sabouraud Medium		Thioglycolate Medium	
	No. of Cultures	Yeast Present	No. of Cultures	Yeast Present	No. of Cultures	Yeast Present
Normal finding ..	668	185	671	363	602	284
Thick stabs	186	40	187	81	168	63
Long stabs	44	12	44	17	43	16
Thin stabs	31	12	31	18	28	11
Indefinite stabs ..	35	8	36	11	35	8
Yeast.....	27	23	27	25	23	20
Yeast-like	33	8	33	13	31	11
No data; preparation poor	13	5	13	7	13	6
Total	1037	293 26.8%	1042	535 50.2%	943	419 43.4%

The direct preparation from the saliva was found serviceable only in the cases in which oval blastospores were clearly visible. It serves as an orientative sample, but if used alone without corresponding cultures it will not give a true picture of the occurrence of yeast fungi.

The findings made in the cultures on different media are shown in table 1. As will be observed, the oil-Sabouraud medium proved superior in sensitivity and gave a positive finding in 50.2 per cent of the cultures, as compared to 43.4 per cent obtained on the anaerobic culture medium and 26.8 per cent on ordinary Sabouraud.

Discussion of Results and Determination of Fungus Status. — To obtain an idea of the amount of fungi in the saliva of each patient we grouped the results according to the following criteria:

Group 0 = Obviously clear cases in which the cultures contained no yeast-like fungi and the native direct preparation was also clean;
Tr (Trace) Group = Some cultures contained 1—2 fungus foci, but not in all types of culture;
Group I = A slight finding of 1—2 foci in all types of culture;
Group II = Up to 10 foci in all culture types;
Group III = Over 10 foci in all types of culture.

Statistical treatment of the findings in table 1 classified according to the above distribution gave the following result[1]:

When the intensity of growth of the yeast = k, and the number of all cultures made on successive days = a, then the amount of growth = b.

$$k = b/a \qquad (0 \leq k \leq 1).$$

1. Distribution 0, Tr., I—III.

	(0)	Tr.	I	II	III
Arithmetic mean of k.	(0)	0.20	0.49	0.72	0.88
Standard deviation of k.	(0)	0.20	0.23	0.24	0.29
Number of cases	68	38	53	43	23

The difference between the groups was highly significant. The deviations in the different groups were the same.

This distribution was a very successful one.

[1] We acknowledge gratefully the kind assistance of Mr. O. Lokki, Ph. D., in connection with the statistical treatment of the results.

TABLE 2

FUNGUS CULTURES CLASSIFIED BY AGE OF PATIENTS

Age	0		Tr.		I		II		III		Total	
	No.	Mean Age	No.	Mean Age	No.	Mean Age	No.	Mean Age	No.	Mean Age	No.	Mean Age
A. Male Patients												
15—20	2	18.0	1	15.0	3	19.3	2	17.5	—	—	8	18.0
21—30	11	24.4	5	27.0	—	—	3	23.7	1	27.0	20	25.1
31—40	5	35.6	1	31.0	10	34.3	4	34.0	1	32.0	21	34.3
41—50	6	45.8	7	45.0	5	46.6	2	44.5	1	47.0	21	45.7
51—60	7	53.7	1	56.0	5	53.4	2	57.0	2	56.0	17	54.4
61—70	2	67.0	1	61.0	1	69.0	2	65.0	2	64.5	8	65.4
71—	—	—	—	—	—	—	1	72.0	—	—	1	72.0
Total	33	38.4	16	38.3	24	41.8	16	40.4	7	49.6	96	40.4
B. Female Patients												
15—20	3	17.7	3	18.7	4	18.5	2	19.0	2	18.5	14	18.4
21—30	13	24.5	1	24.0	3	24.3	3	26.3	—	—	20	24.8
31—40	5	34.4	6	34.5	8	36.1	5	34.6	2	38.0	26	35.3
41—50	3	43.0	5	44.0	5	45.6	3	44.0	3	43.3	19	44.2
51—60	3	55.3	4	53.8	5	54.4	6	56.2	4	56.3	22	55.2
61—70	6	64.0	3	62.7	4	64.5	4	67.8	4	64.0	21	64.6
71—	2	73.5	—	—	—	—	4	75.5	1	77.0	7	75.1
Total	35	39.1	22	41.4	29	41.2	27	49.3	16	50.1	129	43.5
C. Males and Females												
15—20	5	17.8	4	17.8	7	18.9	4	18.3	2	18.5	22	18.3
21—30	24	24.5	6	26.5	3	24.3	6	25.0	1	27.0	40	24.9
31—40	10	35.0	7	34.0	18	35.1	9	34.3	3	36.0	47	34.8
41—50	9	44.9	12	44.6	10	46.1	5	44.2	4	44.3	40	45.0
51—60	10	54.2	5	54.2	10	53.9	8	56.4	6	56.2	39	54.9
61—70	8	64.8	4	62.0	5	66.5	6	66.8	6	64.2	29	64.8
71—	2	73.5	—	—	—	—	5	74.8	1	77.0	8	74.8
Total	68	38.8	38	40.1	53	41.5	43	46.0	23	49.9	225	42.2

Correlation between Sex and Age and the Fungus Status. — A comparison of the fungus status and the sex of the patients revealed no preponderance of either sex (tables 2 A, B and C). On the other hand, the mean age of the patients and the incidence of fungi appeared to have some correlations, for with an increase in the mean age the amount of fungi also increased. Thus in the fungus-free group the mean age of the patients was 38.8 years,

whereas in group III, in which the fungus flora was largest, the mean age was 49.9 years.

Physical Condition and the Fungus Status. — The number of persons in a good or moderately good physical condition was much smaller in group III than in group 0. With an impairment in the general condition the incidence became reversed, and patients in a poor condition fell most frequently into groups II and III (table 3).

TABLE 3

FUNGUS CULTURES CLASSIFIED BY GENERAL PHYSICAL CONDITION OF PATIENTS

Fungus Status	Physical Condition							
	Good		Fair		Poor		Total	
0	32	39.5	28	35.4	7	12.5	67	31.0
Tr.	14	17.3	12	15.2	11	19.7	37	17.1
I	19	23.4	18	22.8	15	26.8	52	24.1
II	10	12.3	16	20.2	14	25.0	40	18.5
III	6	7.4	5	6.3	9	16.1	20	9.3
Total	81		79		56		216	

TABLE 4

FUNGUS CULTURES CLASSIFIED BY DENTAL CONDITION OF PATIENTS

Fungus Status	Dental Condition							
	Good		Fair		Poor		Total	
0	12	66.6	23	34.4	26	21.3	61	32.4
Tr.	3	16.6	15	22.4	19	15.6	37	19.7
I	1	5.6	15	22.4	35	28.7	51	27.1
II	1	5.6	10	14.9	26	21.3	37	19.7
III	1	5.6	4	6.0	16	13.1	21	11.2
Total	18		67		122		207	

Dental Condition and Fungus Status. — Confirmation was obtained for the finding made previously by other workers (31) that the mouth flora contains relatively more fungi when the dental conditions are poor (table 4).

Febrile Conditions, SR and Fungus Status. — As is observed from table 5, a febrile or afebrile condition appears to have no bearing on the fungus status. The mean sedimentation rate, however, was considerably higher in the group of persons with a poor fungus

status. However, this finding is on the whole in harmony with the observation made in connection with the subject's general physical condition.

TABLE 5

FUNGUS CULTURES CLASSIFIED BY FEBRILE CONDITION, SEDIMENTATION RATE AND LEUKOCYTE COUNT OF PATIENTS

Fungus Status	Fever				SR		Leukoc.		Eos.		Mon.		Bas.	
	+		—		No.	Mean	No.	Mean	No.	Mean	No.	Mean	No.	Mean
0	19	27.1	49	31.6	68	28	68	6479	68	2.7	68	6.6	68	0.35
Tr.	12	17.1	26	16.8	37	37	38	7446	38	2.5	38	6.1	38	0.36
I	19	27.1	34	21.9	53	33	51	9741	51	2.6	51	5.6	51	0.28
II	13	18.6	30	19.4	43	30	43	14612	43	2.3	43	6.0	43	0.40
III	7	10.0	16	10.7	22	42	23	5852	23	2.7	23	4.7	23	0.35
Total	70		155		223	33	223	8893	223	2.6	223	6.0	223	0.35

Differential White Cell Count and the Fungus Status. — Table 5 also shows the mean percentages of eosinophils, monocytes and basophils in the leukocyte count. There is a rise in the mean leukocyte count from about 6,000 to 14,000 as the incidence of fungi increases from group 0 to group II, whereas it is lower (about 5,000) in group III, in which the number of fungi was highest. The explanation may be that the patients in this group were physically in the poorest condition, in which the leukocyte reaction was inhibited so that the patient was unable to react in the proper way to the profuse fungus flora. The mean percentage of eosinophils was approximately the same in all fungus groups and it would therefore seem that an increase in the fungus flora does not cause a rise in the number of these cells. The same observation may also be made in respect to monocytes and basophils.

Disease Groups and the Fungus Status. — A rough distribution of the patients according to their principal pathological condition (table 6) indicates no correlation between the diseases and the fungus status except in the small group of patients with diabetes, in which the even distribution of the cases seems to point to a greater incidence of fungi than in the other disease groups. The same tendency appears to be typical of the carcinoma group also.

Antibiotic Therapy and Fungus Status. — To obtain an answer to our second question we carried out new fungus determinations 3

TABLE 6

FUNGUS CULTURES CLASSIFIED BY DISEASES OF PATIENTS

Fungus Status	Carcinoma	Infection	Pulmon. Diseases	Diabetes	Heart Diseases	Digestive Diseases	Total
0	5	34	12	6	21	11	89
Tr.	6	13	4	2	13	7	45
I	7	16	4	4	19	15	65
II	7	15	11	4	17	6	60
III	3	16	3	4	5	2	33
Total	28	94	34	20	75	41	292

weeks later on all patients who had been under treatment at the hospital for 3 weeks or more. This was possible in less than one-half of the original 292 cases, either because of a shorter period of hospitalisation or because a sufficient number of cultures could not be made for various reasons. Table 7 shows the change in the fungus status of the 123 patients for whom the determinations were made at both times.

TABLE 7

CHANGES IN FUNGUS STATUS DURING 3 WEEKS' HOSPITAL TREATMENT

Fungus Status	No Antibiotics	Penicillin	Wide Spectric Antibiotics	Total
Improvement	7	7	3	17
No change	30	28	8	66
Aggravation	14	14	12	40
Total	51	49	23	123

The penicillin dosages varied from 4.2 million to 16 million units. The wide spectric antibiotics administered were aureomycin, chloromycetin, terramycin or streptomycin in doses of 20—30 gm. It is observed from the table that a change occurred in the fungus status in less than 50 per cent of the cases only.

The improvement in the fungus status was not greater than one degree in any of the cases. On the other hand, aggravation of the status by more than one degree occurred in 13 patients, only 2 of whom had not received antibiotics, 7 had been administered penicillin and 4 wide spectric antibiotics.

Comparison of the non-antibiotic group and the penicillin group shows that treatment with penicillin in the dosages given probably

has no effect on the occurrence of fungi in saliva. The number of patients who received wide spectric antibiotics was so small that no definite conclusion can be drawn regarding its effect, although there were signs of an increase in the number of yeast-like fungi in the salivary flora.

DISCUSSION AND SUMMARY

The occurrence of yeast-like fungi in the human saliva is greatly variable also in persons in whom there are no signs of infection by fungi. In our study, yeast-like fungi were found in the saliva of 27—50 per cent of ordinary hospital patients, the variations being probably due to differences in the sensitivity of the methods and in the culture media used. One may therefore be justified in saying that certain yeast-like fungi belong, in a way, to the »normal flora» of saliva. It was seen in our study that the occurrence and amount of yeast-like fungi in the saliva may be specified by classification of the subjects into, for instance, five groups. The correctness of this classification was borne out by the statistical treatment of the results in our series of cases. The data listed in the tables also indicated that the distribution of the cases into these groups had some degree of correlation to certain known features in the cases. For instance, the incidence of fungi is higher in persons of advanced age, with poor dental conditions, or in a weak physical condition, than in young persons and those in a good physical condition.

On the other hand, the incidence of fungi showed no definite correlation to other features in the clinical picture or in the routine blood analysis, and thus there was no certain finding that would be an aid in the determination of the presence and amount of yeast-like fungi. This is quite comprehensible, since the saprophytic flora has not been found to produce generally any demonstrable symptoms in its host.

There is evidence to justify the statement that accuracy in the determination of the fungus status cannot be obtained by merely one routine examination of the saliva samples. Several consecutive cultures will give a better picture of the changes possibly occurring in the fungus status of the patient. Thus the section of our study relating to the effect of antibiotic treatment on the fungus flora clearly indicated that the usual *penicillin treatment given over a*

relatively short period in medical hospitals has no greater effect on the fungus status, as determined from the saliva, *than other hospital therapy.* In our small series there were, *however, findings pointing to an increase in the fungus flora in the saliva after routine administration of wide spectric antibiotics.* For this reason it is to be recommended that during prolonged treatment with wide spectric antibiotics attention should also be paid, by means of repeated cultures, to possible changes in the flora of yeast-like fungi in the patient's saliva.

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FROM THE DEPARTMENT OF BACTERIOLOGY AND SEROLOGY, UNIVERSITY OF TURKU

HEMOPHILUS PERTUSSIS

SENSITIVITY IN VITRO TO EIGHT ANTIBIOTICS AND SULPHATHIAZOLE

by

SIMO VIRTANEN

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The present paper deals with studies on the *in vitro* sensitivity of newly isolated strains of *H.pertussis* to carbomycin, chloramphenicol, chlortetracycline, neomycin, oxytetracycline, penicillin, polymyxin B, streptomycin and sulphathiazole. The results of previous investigations have been somewhat variable, especially regarding chlortetracycline (1, 2, 4, 5, 6).

MATERIAL AND METHODS

The investigated strains were obtained from nasopharyngeal swab cultures from active cases of whooping cough. Bordet-Gengou medium containing 30 per cent fresh, defibrinated horse blood was used for the primary isolation and the sensitivity tests. The organisms were identified by their colony and morphological characters and by slide agglutination in specific antisera. All the strains studied remained in the S-phase during the tests.

The drug sensitivities were evaluated by the plate dilution method. The strain under test was incubated for 48 hours in Verwey liquid medium (7). The suspension was used immediately after dilution to 1:20 with the same medium. One loopful of the diluted suspension was seeded on the plates to obtain non-confluent colonies.

The cultures were incubated at 37°C. The sensitivity of each organism was defined by giving the minimum concentration of antibiotic necessary to effect complete inhibition of growth after 72 hours. Standard strains were included in each test to control the activity of the antibiotics and the validity of the tests. With the exception of neomycin, the antibiotics employed were from antibiotic kits donated by Pfizer & Co. The neomycin was made available by Lääke Oy., Turku. A 20 per cent sulphathiazole solution (Ciba) was employed when studying the action of this drug.

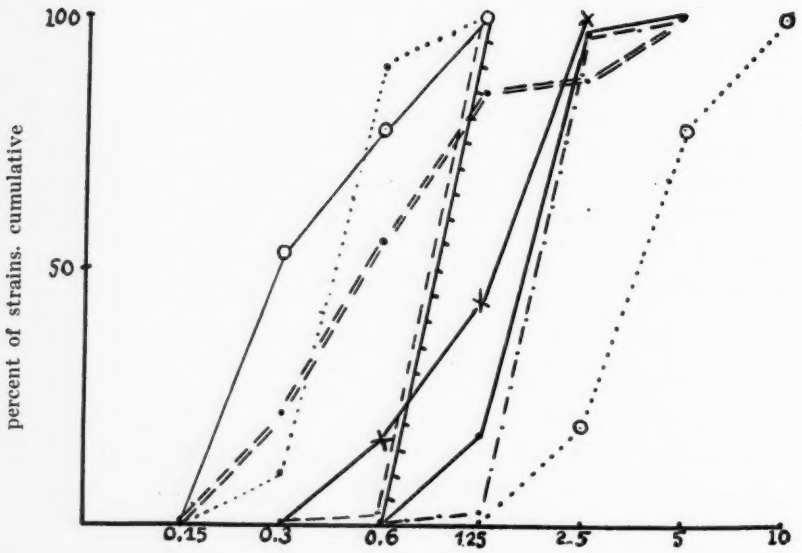
RESULTS

The results obtained are presented in Table 1 and Fig. 1. It is evident that none of the strains tested was completely resistant to the therapeutic agents. The fairly high values obtained with chlortetracycline are evidently partly due to its rapid decomposition during the incubation (3). *Hemophilus pertussis* is fairly sensitive to penicillin (6); 2.5 I.U. of penicillin per ml. inhibited the growth of 40 of the 41 strains tested and even 1.25 I.U./ml. retarded the growth considerably. This latter fact should be taken into account when using a medium containing penicillin to isolate *Hemophilus pertussis* strains. It has been reported that *H. pertussis* is insensitive to sulpha drugs. The strains of the present study were all found sensitive to sulphathiazole in vitro although the

TABLE 1

THE SENSITIVITY OF HEMOPHILUS PERTUSSIS STRAINS TO 8 ANTIBIOTICS AND SULPHATHIAZOLE

Therapeutic Agent	Inhibition Concentration for Penicillin in I.U./ml, for Others in Microg/ml						No. of Strains Examined
	10	5	2.5	1.25	0.6	0.3	
Carbomycin			23	11	7		41
Chloramphenicol				9	10	22	41
Chlortetracycline	9	24	8				41
Neomycin				40	1		41
Oxytetracycline		1	33	7			41
Penicillin		1	39	1			41
Polymyxin B ..				41			41
Streptomycin ..				4	33	4	41
Sulphathiazole..		5	1	12	14	9	41



Minimum complete inhibition concentration for penicillin in I.U./ml; for others in migrog/ml

x——x Carbomycin
o——o Chloramphenicol
o·····o Chlortetracycline
Polymyxin B
------ Neomycin
Oxytetracycline
·—·—· Penicillin
· ····· · Streptomycin
· ==== · Sulphathiazole

Fig. 1. — Antibiotic spectrum of Hemophilus pertussis.

culture medium contained sulphonamide inhibitors. A comparison of the inhibition concentrations for 10 bacterial strains (6 staphylococci, 3 E. coli and 1 S. paratyphi strains) in peptone-free blood agar medium (8) and in Bordet-Gengou medium revealed that the inhibition concentrations of sulphathiazole were four times higher in the latter medium.

SUMMARY

The drug sensitivities *in vitro* of 41 *Hemophilus pertussis* strains have been examined. All these strains were found to be resistant to carbomycin, chloramphenicol, chlortetracycline, neomycin, oxytetracycline, penicillin, polymyxin B, streptomycin and sulphathiazole. The strains were moderately sensitive to penicillin. The lowest inhibition concentrations were obtained for chloramphenicol and streptomycin.

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FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI

ETHER SIMULATING ALCOHOL IN BLOOD

STUDIES ON MEDICAL STAFF ENGAGED IN AN OPERATION PERFORMED UNDER ETHER ANESTHESIA

by

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(Received for publication October 23, 1954)

Widmark's micromethod is the only method used in Finland for medico-legal determination of the blood alcohol (1, 9). The method, as known, is based on an oxidation-reduction reaction which is non-specific to ethyl alcohol. Several other reducing substances present in the blood, such as ether, may show elevated values when the method is applied.

Recently practitioners inquired at the Institute of Forensic Medicine whether in the course of operations carried out under ether anaesthesia such amounts of ether might possibly pass into the blood of the persons engaged in an operation as to be of medico-legal significance. As no answer was to be found in the literature, it was thought justifiable to undertake an investigation of the matter.

By Widmark's micromethod a low reduction value is obtained on the blood of abstainers, 0.03 per mil on an average according to older studies (4). According to Goldberg (8) the value exceeds 0.04 per mil in only 2.5 per cent of cases, and in 0.5 per cent it is over 0.06 per mil. This value may be mainly due to analytical errors of a technical nature, but possibly also to minimal amounts of evaporating reducing compounds (so-called endogenous, »physiological», blood alcohol) in the blood. In a medico-legal

sense, however, the mean error was estimated at 0.05 per mil; in Finland, tor instance, in determinations carried out for medico-legal purposes, a friple mean error — 0.15 per mil — is subtracted from the mean value of two or three parallel analyses.

Widmark's method is also applicable for quantitative determination of the ether content of blood. Compared with alcohol determinations the method involves a technical difference in calculation: while the constant corresponding to the thiosulfate equivalent is 1.13 for ethyl alcohol, it is 0.9—0.92 for ether (2, 3).

According to the literature the ether content of blood varies between 1.0 and 1.4 per mil under complete ether anaesthesia. An ether concentration rising above 1.7—1.85 per mil makes the condition fatal (6, 7). According to Mueller (5), with the ethyl alcohol constant at 1.13 values between 0.69 and 1.52 per mil were obtained for conditions ranging from an incipient awaking to a deep narcosis.

The well-known pharmacological fact that ether becomes eliminated very rapidly and almost without decomposing, mainly by way of the lungs, should also be mentioned; small amounts are secreted into the urine and through the skin (6).

The study was carried out at the Department of Forensic Medicine, University of Helsinki, the Second Women's Clinic, and Children's Clinic in January—in April 1954. Twenty-six subjects in all were examined, both operating surgeons and nurses administering open ether anesthesia. Blood samples were taken from them during the operations while the patient was under complete narcosis. Thirty-two samples were obtained from 22 subjects altogether while the Julliard mask was on and 4 samples from 4 subjects while the dropping mask was applied.

The blood samples were taken from the vein direct into two Widmark capillaries. The analyses were determined within 24 hours of the taking of the specimens. 1.25 g/500 ml of $K_2Cr_2O_7$—H_2SO_4 solution and 1/100 n of $Na_2S_2O_3$ solution were used in all the determinations. In calculating the results the ethyl alcohol constant 1.13 was used. The reduction value was calculated as a mean of two parallel analyses. In 22 determinations the parallel values tallied. For the rest the largest difference was 0.038 per mil. The highest value obtained from one single capillary was +0.046 and the lowest —0.019 per mil. The distribution of the reduction values and the means appear from the Table 1.

The results show that in most of the cases the reduction value obtained was ± 0. Only in one case did the value exceed 0.04 per

TABLE 1

DISTRIBUTION OF REDUCTION VALUES, AND MEAN VALUES

	Number of Determinations, ‰								
	0.019 - 0.007	±0	0.00 - 0.01	0.01 - 0.02	0.02 - 0.03	0.035	0.046	Total Number of Determinations	Mean Values
Julliard's mask..	1	18	4	4	4	1	—	32	+0.0069 per mil
Instillation mask	1	2	—	—	—	—	1	4	+0.0100 » »
The whole material								36	+0.0073 per mil

mil but not 0.05 per mil. Thus, for open ether narcosis induced under ordinary conditions in Finland, no certain elevated value was obtained.

It seems from the study performed that under administration of ether no such quantities of ether pass into the blood of the hospital staff participating in an operation as to give the matter any medico-legal significance, when the determination of the blood alcohol is carried out by Widmark's micromethod.

SUMMARY

Using both Julliard's mask and the dropping mask in inducing of open ether anaesthesia, 36 blood samples in all were taken from 26 subjects of the hospital staff participating in an operation, and the reduction value was calculated by Widmark's micromethod. No elevated value was obtained with certainty in any of the cases. The mean value for the whole of the series was 0.0073 per mil and the limit values were —0.019 and +0.046 per mil.

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FROM THE SIR WILLIAM DUNN SCHOOL OF PATHOLOGY, UNIVERSITY OF OXFORD

THE BACTERIOSTATIC EFFECT OF ERYTHROMYCIN-STREPTOMYCIN COMBINATIONS

by

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Streptomycin and erythromycin in combination are reported to depress the development of resistance of bacteria to these antibiotics (6). Combinations of streptomycin and aureomycin, chloromycetin or terramycin are found to react in the same way (5). But these combinations are rather antagonistic in their bactericidal action (4), as are also streptomycin and erythromycin combined (1). A review of the numerous conflicting studies, theoretical and clinical, of the combined effect of antibiotics is given by Garrod, 1953 (2).

The purpose of the present work was to study *the bacteriostatic effect of streptomycin and erythromycin in combination*. Bacteriostatic action was determined by the vertical diffusion method. The following medium, based on »Difco» streptomycin assay agar, was used:

Beef extract (Lemco)	0.5 g.
Yeast autolysate (Glenochil)..	7.0 g.
»Bacto» peptone	3.0 g.
» agar	4.5 g.
Distilled water	300.0 ml.

Adjust reaction to pH 8.0: autoclave.

The following test organisms were employed:

Staph. aureus »P», strain freshly isolated from a patient
» » »O», laboratory strain
E. coli, » »
β-haemolytic streptococcus, laboratory strain.

The agar was kept melted at 45°C in a water-bath. An overnight broth culture of the test organism was added to the agar making a final dilution of 10^{-4}. Tubes 3 mm in diameter were filled to a depth of approximately 5 cm. with the inoculated medium. After solidification the inoculated medium was overlaid to a depth of approximately 1 cm. with different serial dilutions of streptomycin in Sørensen's M/15 phosphate buffer, pH 8.0. After 24 hours at 37°C the length of the zone of growth inhibition was measured with a low power travelling microscope and vernier scale. Taking the average of 4 replicates for each concentration, a standard reference curve of streptomycin concentration/length of zone of inhibition was prepared (Fig. 2).

The effect of streptomycin when combined with erythromycin was measured as described above. The erythromycin was dissolved in the medium. The media were prepared with the following concentrations of erythromycin for measurement of the bacteriostatic action of streptomycin:

— agar without erythromycin
— » with the minimum growth-inhibiting concentration (M.I.C.) of erythromycin
— » » 1/2 M.I.C. of erythromycin
— » » 1/4 » » »
— » » 1/8 » » »

Reference curves of streptomycin concentration/length of zone of inhibition in erythromycin-containing agar were prepared as the standard curve (Fig. 2).

The zone of inhibition in the agar indicating the bacteriostatic action of erythromycin was determined by a similar technique; i.e. the erythromycin solution was on the agar surface, the streptomycin dissolved in the agar in the minimum growth inhibiting concentration (M.I.C.), 1/2, 1/4 and 1/8 M.I.C.

Fig. 1 shows the series comprising the different zones of inhibition produced by streptomycin on the agar surface and by the different amounts of erythromycin in the agar. Series II has the M.I.C. of erythromycin in the agar, for which reason it could be assumed that no growth occurred unless erythromycin had an antagonistic effect on streptomycin. Staph. aureus strain »O» showed weak growth in this series. The growth appeared in an

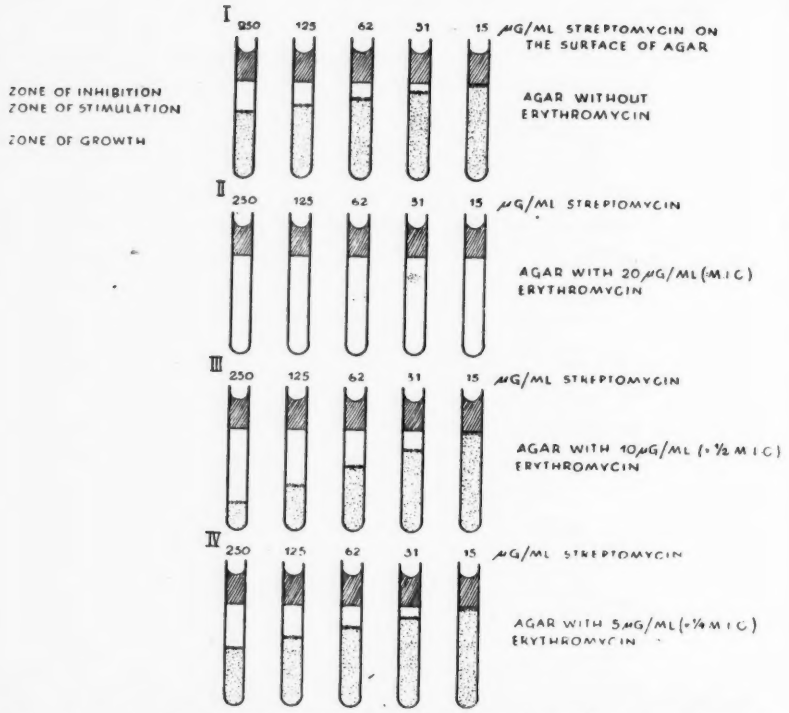
I
250 125 62 31 15 µG/ML STREPTOMYCIN ON THE SURFACE OF AGAR
ZONE OF INHIBITION
ZONE OF STIMULATION
ZONE OF GROWTH
AGAR WITHOUT ERYTHROMYCIN
II
250 125 62 31 15 µG/ML STREPTOMYCIN
AGAR WITH 20µG/ML (M.I.C.) ERYTHROMYCIN
III
250 125 62 31 15 µG/ML STREPTOMYCIN
AGAR WITH 10µG/ML (1/2 M.I.C.) ERYTHROMYCIN
IV
250 125 62 31 15 µG/ML STREPTOMYCIN
AGAR WITH 5µG/ML (1/4 M.I.C.) ERYTHROMYCIN

Fig. 1. — Vertical diffusion method used to show the action of erythromycin (in subinhibiting concentrations) on the bacteriostatic effect of streptomycin. Minimum growth-inhibiting concentration = M.I.C.

area corresponding to the zone of stimulation in those tubes, e.g. in Series I, in which a zone of stimulation formed between the zone of growth and zone of inhibition. With higher streptomycin concentrations (Series I) the zone of stimulation was less clear, and in the corresponding tubes in Series II no growth was observable. In Series III, with 1/2 M.I.C. of erythromycin in the agar, there was a marked increase in the zone of inhibition of streptomycin compared with the corresponding tubes of standard Series I. The increase was least in low streptomycin concentrations, rising gradually in the higher streptomycin concentrations. In Series IV, with 1/4 M.I.C. of erythromycin, the increased zone of inhibition was smaller than in Series III but still observable. With 1/8 M.I.C. of erythromycin in the agar the zone of inhibition was approximately the same as in the standard series without erythromycin.

Erythromycin increased the bacteriostatic action of strepto-

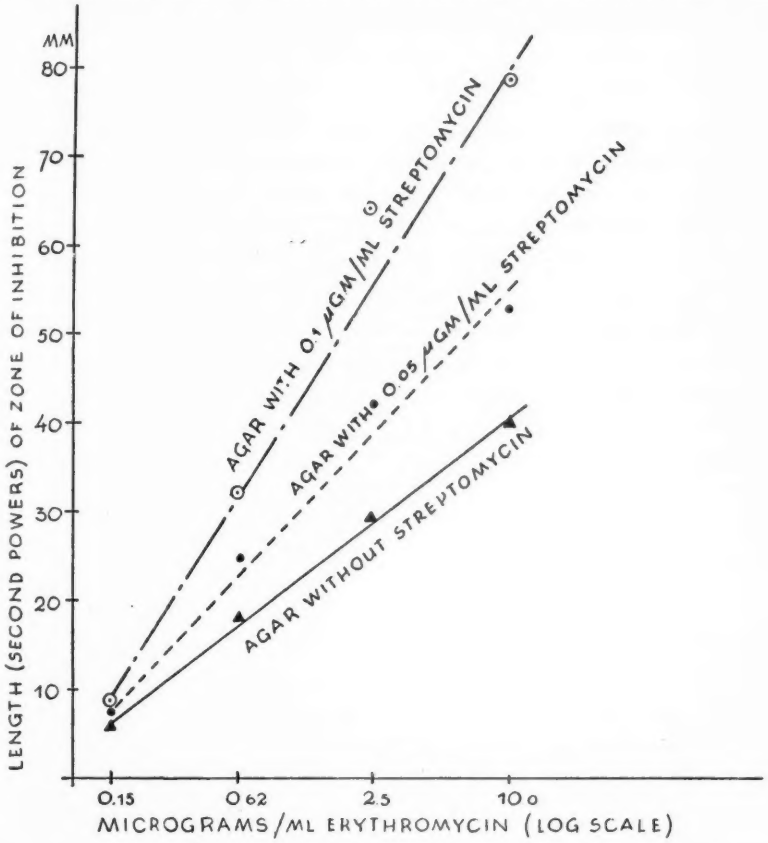

Fig. 2. — Reference curves indicating length of zone of inhibition / concentration of erythromycin in agar without streptomycin and with different sub-inhibiting concentrations of streptomycin. Test organism: Staph.aureus »O».

mycin on all strains other than the β-haemolytic streptococcus strain.

In tests with *streptomycin in the agar and erythromycin on the surface* streptomycin increased the erythromycin zone of inhibition with all the strains employed, least, however, with the β-haemolytic streptococcus strain, with which only 1/2 M.I.C. of streptomycin in the agar gave an observable increase in the zone of inhibition. The increase in the zone of inhibition of erythromycin is illustrated by the fact that in tests using Staphylococcus aureus strain »P», 0.5 μg/ml of erythromycin on the surface of the agar together

with 1/2 M.I.C. of streptomycin in the agar gave a zone of inhibition of the same size as 30 μg/ml of erythromycin alone.

It is a known fact that the zone of inhibition of erythromycin can be increased by making the pH of the agar more alkaline (3). When this is done the minimum concentration of erythromycin giving a zone of inhibition falls with rise in alkalinity. It may be seen from the present study that the addition of streptomycin to the agar does not give a corresponding increase. The *smallest amount of erythromycin producing a zone of inhibition remains the same even when streptomycin is added to the agar* (Fig. 2). The reference curves of the zone of inhibition of erythromycin at two different pHs were found to run a parallel course. This was in contrast to Fig. 2 in which they take a divergent course from a common starting point.

SUMMARY

1. Using the vertical diffusion method, it was found that erythromycin added to agar in subinhibiting concentrations increased the bacteriostatic action of streptomycin if the streptomycin concentration was high enough to give a zone of inhibition even without erythromycin. The increase was greatest with half the minimum growth inhibiting concentration (1/2 M.I.C.) of erythromycin in the agar. 1/4 M.I.C. of erythromycin gave a slight increase but 1/8 M.I.C. failed to give any increase at all. This combined action was ascertainable with two strains of Staphylococcus aureus and one of E. coli, but not with the strain of β-haemolytic streptococcus used.

2. The streptomycin present in the agar correspondingly increased the bacteriostatic action of erythromycin against all the strains used, but least against the β-haemolytic streptococcus strain.

3. The minimum concentration of the antibiotic on the surface of the agar giving a zone of inhibition was the same irrespective of whether the other antibiotic was present as a component of the agar or not.

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FROM THE STATE SERUM INSTITUTE, HELSINKI

SERUM CONCENTRATION OF DIFFERENT COMMERCIAL PENICILLIN PREPARATIONS

II

PREPARATIONS CONTAINING PROCAINE AND SODIUM PENICILLIN G IN AQUEOUS OR OILY SUSPENSION, AND BENZYLPENICILLIN-β-DIETHYLAMINOETHYLESTER HYDROIODIDE

by

VEIKKO TOMMILA and TAPIO SAVOLAINEN

(Received for publication October 28, 1954)

In our previous tests (13, 14), it was demonstrated that the results obtainable with different aqueous and oily suspensions of procaine penicillin are greatly divergent, and that some oily suspensions gave such low serum penicillin concentrations that they were unsuited for the treatment of hospital patients. We have, therefore, made further tests which include the so-called dipenicillins, *i.e.*, preparations that in addition to procaine penicillin contain sodium penicillin. Observations were made on the serum penicillin concentrations during 24 hours.

MATERIAL

The tests series consisted of 15 commercial preparations for injection that contained penicillin only. According to the manufacturers' declarations, 75 per cent by unit of most of the products was procaine penicillin G and 25 per cent was sodium or potassium penicillin G; one additional test preparation was benzylpenicillin-β-diethylaminoethylester hydroiodide.

For this study, which was carried out in 1952—1954, purchases were made from different pharmacies and pharmaceutical wholesalers, by the usual procurement procedure of practitioners, of all available preparations

for injection treatment that contained procaine penicillin G and sodium or potassium penicillin G and were stated by the manufacturers to be effective. A few additional preparations were procured from the manufacturers, either directly or through their agents.

CASE SERIES

The test subjects were adults hospitalised in the Helsinki University Hospital for Ear, Nose and Throat Diseases and in the First Central Military Hospital.[1] The age of the patients ranged from 17 to 65 years and their body weights from 46 to 82 kg. Each test subject was given an intramuscular injection into the gluteal region of 600.000 units of the penicillin preparation under investigation, except for benzylpenicillin-β-diethylaminoethylester hydroiodide, of which 500.000 units were given. Blood samples were taken immediately before the injection and 1, 2, 3, 6, 12 and 24 hours afterward. Each preparation was tested on several persons (an average of five persons per preparation), and the average of the results was calculated and shown by graph. The determinations were carried out on a total of 83 test subjects.

TECHNIQUE

All the penicillin determinations were performed according to the cylinder plate method of the WHO (16), subject to a few minor deviations (13).

RESULTS

The results are most clearly seen from the graphs showing the mean concentrations, in which the abscissa is the time from the injection and the ordinate the penicillin concentration in the serum. To render the results more comprehensible, the minimum therapeutic concentration is shown in the graph at 0.05 unit per millilitre of serum. The selection of this point is obviously somewhat arbitrary, since the effective therapeutic concentration varies according to the case and the species and strain of the bacterium. In syphilis therapy, for instance, 0.03 u./ml is regarded as the minimum therapeutic concentration (17).

Aqueous Suspensions Containing Procaine Penicillin G and Sodium Penicillin G. — There were altogether 13 preparations which contained procaine penicillin G, usually 75 per cent, and

[1] We gratefully acknowledge the kindness of Professor Y. Meurman, M.D., Chief of the Helsinki University Hospital for Ear, Nose and Throat Diseases, and Major-Gen. P. Somer, M.D., Chief Medical Officer of the Finnish Army, in placing at our disposal a sufficiently large series of patients.

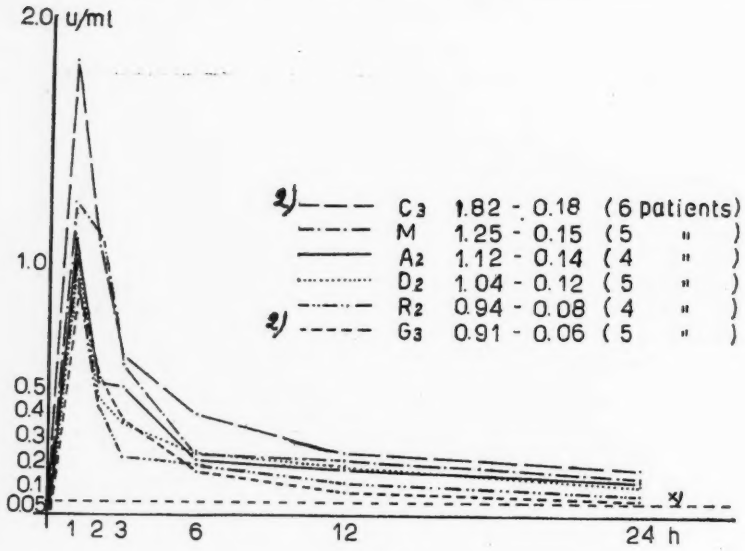

Fig. 1.

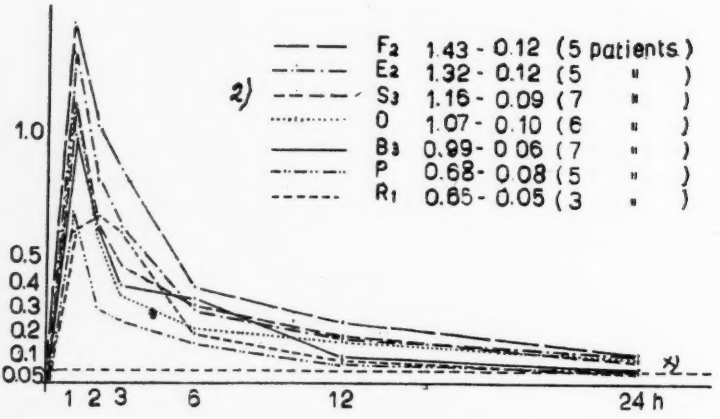

Fig. 2.

Figs. 1 and 2[1]. — Average penicillin concentrations in sera of adults during 24 hours after a single intramuscular injection of 600.000 units of various commercial preparations containing 75 per cent of procaine penicillin G (67 per cent in one preparation) and 25 per cent of sodium (or potassium) penicillin G (33 per cent in one preparation) in aqueous suspension.

* Minimum therapeutic concentration.

1) The preparations are designated by letters; small figures indicate the numbers of the products of each manufacturer. In the following columns are given the mean maximum concentration (in units per ml of serum) obtained with each preparation and the mean concentration (in units per ml of serum) 24 hours after the injection.

2) In our determinations, the penicillin contents of preparations C_3, G_3 and S_3 were 300.000—360.000, 270.000—300.000 and 285.000 u./ml, respectively.

sodium (or potassium) penicillin G, usually 25 per cent, in aqueous suspensions. The results are shown in Figs. 1—3 and table 1.

In Figs. 1 and 2 the results are given in the form of curves for the mean values, which can best be evaluated if we know the individual variations in the results for the different patients after administration of the same product and the standard deviations of the mean values. Fig. 3 shows the penicillin concentrations for all the patients who received either preparation B_3 or A_2. Of the 13 tested procaine and sodium penicillin products in aqueous suspension, preparation B_3 gave the greatest and A_2 the smallest individual variations. For the other products, the individual variations were within these limits. Table 1 shows the standard deviations of the mean values for the maximum concentrations and for the concentrations at 24 hours.

TABLE 1[1]

TESTED PREPARATIONS CONTAINING PROCAINE AND SODIUM PENICILLIN G IN AQUEOUS OR OILY SUSPENSION, NUMBER OF PATIENTS, MEAN SERUM PENICILLIN CONCENTRATIONS AND STANDARD DEVIATIONS AT MAXIMUM CONCENTRATION AND 24 HOURS

Product	No. of Patients	Mean Maximum Concentration		Mean Concentration at 24 Hours	
		u./ml. of Serum	Its Standard Deviation	u./ml. of Serum	Its Standard Deviation
C_3	6	1.82	±0.13	0.18	±0.04
F_2	5	1.43	±0.21	0.12	±0.05
E_2	5	1.32	±0.18	0.12	±0.03
M	5	1.25	±0.33	0.15	±0.03
S_3	7	1.16	±0.23	0.09	±0.01
A_2	4	1.12	±0.08	0.14	±0.01
O	6	1.07	±0.10	0.10	±0.02
D_2	5	1.04	±0.23	0.12	±0.04
B_3	7	0.99	±0.22	0.06	±0.02
R_2	4	0.94	±0.31	0.08	±0.05
G_3	5	0.91	±0.32	0.06	±0.02
P	5	0.68	±0.20	0.08	±0.01
R_1	3	0.60	±0.14	0.05	±0.02
S_1	4	0.49	±0.12	0.08	±0.01
S_2	4	0.32	±0.17	0.13	±0.05

[1] We gratefully acknowledge the assistance of Mr. E. Kaila, Ph.D., in checking the mean values and calculating the standard deviations.

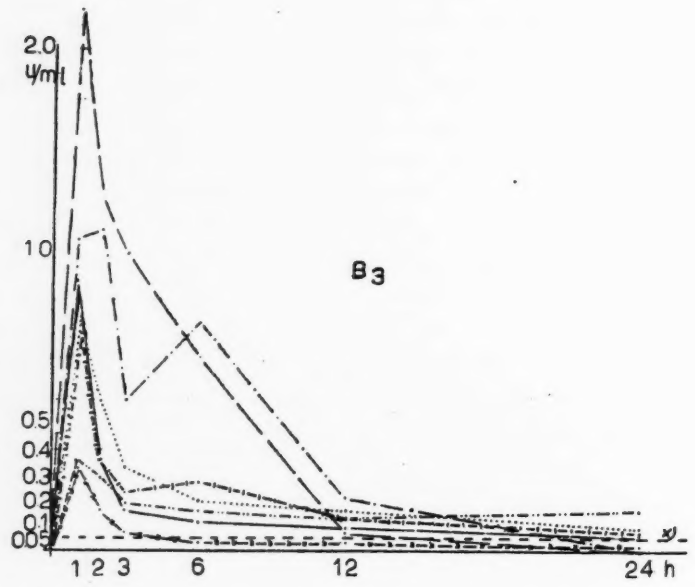

Fig. 3 a.

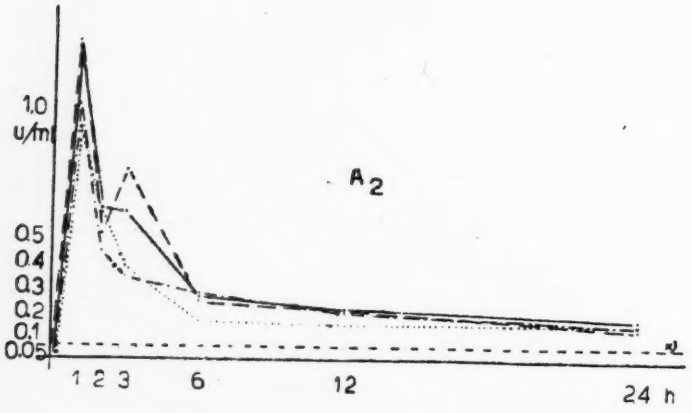

Fig. b.

Fig. 3. — Individual penicillin concentrations in sera of adults during 24 hours after a single intramuscular injection of 600.000 units of preparation B_3 or A_2.

* Minimum therapeutic concentration.

Oily Suspensions Containing Procaine Penicillin G and Sodium Penicillin G. — There were two preparations which contained procaine penicillin G 75 per cent by unit and sodium penicillin G 25 per cent by unit in oily suspensions. The results are given in Fig. 4 and Table 1.

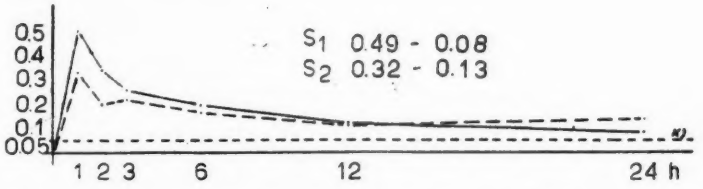

Fig. 4. — Average penicillin concentrations in sera of four adults during 24 hours after a single intramuscular injection of 600.000 units of two commercial preparations containing 75 per cent by unit of procaine penicillin G and 25 per cent sodium penicillin G in oily suspension.

* Minimum therapeutic concentration.

Preparation Containing Benzylpenicillin-β-diethylaminoethylester Hydroiodide. — The results are shown in Fig. 5.

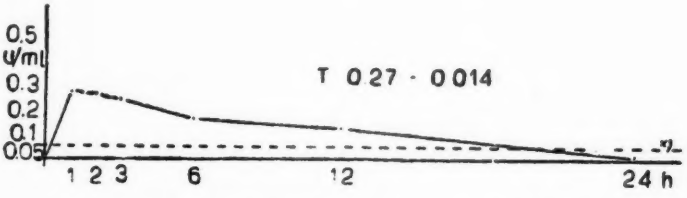

Fig. 5. — Average penicillin concentration in sera of eight adults during 24 hours after a single intramuscular injection of 500.000 units of a commercial preparation containing benzylpenicillin-β-diethylaminoethylester hydroiodide in aqueous suspension.

* Minimum therapeutic concentration.

DISCUSSION

The value of the present investigation lies essentially in the circumstance that these comparisons of different commercial preparations of penicillin were made by the same investigators, by one technique, at one time and with similar series of hospital patients.

The average penicillin concentrations for the different products are shown in the forms of graphs in Figs. 1, 2, 4 and 5. From Fig. 3 are seen the limits within which analogous results are obtained

with the same preparations in different patients. Table 1 shows the standard deviations of the mean values for each product at the time of maximum concentration and at 24 hours after injection.

All the products containing both procaine and sodium penicillin in aqueous suspension were capable of maintaining for 24 hours a serum penicillin concentration in excess of the minimum therapeutic concentration. The high maximum concentrations, *ad* 1.82 u./ml of serum, should particularly be noted.

The corresponding oily suspensions also gave penicillin concentrations which at 24 hours exceeded the minimum therapeutic concentration, but the mean maximum concentrations in particular were relatively low in comparison with those of the preceding group, *i.e.*, lower than the mean maximum concentration of the weakest preparation in aqueous suspension, and the mean concentrations at 24 hours were on a level with the medium high concentrations produced by preparations in the preceding group.

The difference in the maximum concentrations of the various products in aqueous suspension was nearly three-fold (product $R_1 = 0.65$ u./ml as against product $C_3 = 1.82$) and in the 24-hour concentrations more than three-fold (product $R_1 = 0.05$ u./ml as against product $C_3 = 0.18$). The oily suspensions showed 1.5-fold differences at maximum concentration and at 24 hours ($S_2 = 0.32$ u./ml as compared with $S_1 = 0.49$; $S_1 = 0.08$ as against $S_2 = 0.13$). A comparison of all the preparations tested showed a six-fold difference in the maximum concentrations and a four-fold difference in the concentrations at 24 hours. Taking into consideration also the products tested by us earlier (13), the difference in the maximum concentrations was 36-fold and in the 24-hour concentrations more than tenfold.

Only one preparation containing benzylpenicillin-β-diethylaminoethylester hydroiodide was tested. The maximum concentration attained in the serum was 0.27 u./ml, and the concentration at 24 hours was 0.014 (Fig. 5), the practical duration in the serum being about 18 hours. This preparation is, therefore, to be administered at intervals of 12 hours, as was also reported by Jensen *et al.* (7, 8, 9, 10, 11), if it is desired to maintain an assayable penicillin concentration in the serum throughout the treatment. The affinity of benzylpenicillin-β-diethylaminoethylester hydroiodide

for certain organs deserves to be noted, for it becomes concentrated especially in the lungs and it is excreted in an active form in, for instance, the sputum, in concentrations up to 3 u./ml of sputum (2, 4, 7, 8, 9, 10, 11, 12). To a smaller degree it also concentrates in the brain (9). Studies by Anttinen (1), Gylfe *et al.* (6) and Schimmel *et al.* (15) showed its rapid resorption into the cerebrospinal fluid, in which it can be assayed one to two hours after injection (intramuscular) in amounts two to three times the minimum therapeutic concentrations, the duration of the effect being six to eight hours (1, 6).

With all the preparations, the maximum serum concentration was reached rapidly, *i.e.*, in one hour, with the exception of one product, which attained it in two hours.

In view of the differences in the serum concentrations attained with the various penicillin preparations — a circumstance which was also observed in earlier tests (3) — they cannot be considered to be of equal therapeutic value. This variability in the results is evidence that the continuous performance of investigations of this kind is necessary.

The results obtained by us can, of course, be applied in therapeutic practice. Thus, a high penicillin concentration in the blood can be reached and maintained by injection of some of these penicillin preparations at 12-hour intervals; by injection of the other preparations at 24-hour intervals, a relatively high level can be maintained for this period or even longer. The case under treatment and the method of administration employed naturally determine the selection of the preparation and the dosage. In this respect, it has been shown that with larger doses the penicillin concentration in the serum also increases (5). It is self-evident that successful treatment is not dependent on the serum penicillin level alone, but also, among other factors, on the penicillin concentration attained in the diseased organ. The penicillin concentration in the organism is highest in the kidneys and lowest in the brain. In other organs, it usually is considerably lower than in the blood, being, however, higher in an inflamed organ than in a healthy one, provided the former has a good blood circulation (5). Penicillin is retained in assayable amounts in the tissues for a longer time than in the blood. This is one of the reasons that stoss treatment, in which penicillin is no longer discernible in the blood between injections, may give

good therapeutic results, although the results are no better than those obtainable with continuous treatment (5). Since penicillin generally concentrates poorly in cerebral tissue, note should be taken of the concentration of benzylpenicillin-β-diethylaminoethylester hydroiodide in the brain and of its still greater concentration in the lungs. The interesting observation has also been made that penicillin administered during pregnancy is absorbed by the fetus in an amount equalling one-half to one-sixth of the penicillin concentration in the maternal blood (18).

SUMMARY

1. This report covers the results of tests with 16 commercial penicillin preparations consisting of 13 preparations containing procaine penicillin G (75 per cent by unit) and sodium (or potassium) penicillin G (25 per cent by unit) in aqueous suspension; 2 preparations containing procaine penicillin G (75 per cent by unit) and sodium penicillin G (25 per cent by unit) in oily suspension, and one preparation of benzylpenicillin-β-diethylaminoethylester hydroiodide in aqueous suspension. The tests were made on sera from 83 patients by cylinder plate method. A single intramuscular injection of 600.000 units (500.000 units in the case of benzylpenicillin-β-diethylaminoethylester hydroiodide) was given to the subjects, and the penicillin concentration in the serum was determined at various intervals during 24 hours.

2. The results obtained show marked differences among all the preparations tested as well as among the individual preparations within a product group. Thus, the choice of preparation in each case is not unimportant from a therapeutic point of view. The »best» results were obtained with preparations containing both procaine penicillin and sodium penicillin G in aqueous suspension (the »best» product giving a maximum concentration of 1.82 u./ml of serum and a concentration at 24 hours of 0.18 u./ml of serum), whereas the corresponding oily suspensions were weaker. Benzylpenicillin-β-diethylaminoethylester hydroiodide attained a serum concentration that remained above the minimum therapeutic concentration for 18 hours; the maximum concentration was 0.27 u./ml of serum.

3. All the tested preparations attained their maximum serum

concentrations rapidly, usually within one hour, except for one that reached its maximum concentration in two hours.

4. In view of the marked differences observed in the tested preparations, the constant performance of tests of this kind appears to be indispensable.

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OBSERVATIONS ON LIVING THROMBOCYTES UNDER THE ANOPTRAL CONTRAST MICROSCOPE

by

MAIJA-LIISA KOTILAINEN and ALVAR WILSKA

(Received for publication November 13, 1954)

Anyone who is familiar with haematological dry-smear staining technique knows how inconspicuous the blood platelets appear among the erythrocytes and leukocytes. Despite their insignificant appearance, these smallest cytomorphic entities of the human body have important functions. We have reason to assume that many qualities connected with the activity of thrombocytes are only partially known because of the difficulties of observing these cellular midgets under living conditions with sufficient contrast and resolution.

The anoptral contrast microscope, reported on by one of us (1—3) and manufactured by Optische Werke C. Reichert of Vienna, has proved particularly suitable for this purpose. Many details of the thrombocytes which are almost or totally invisible with ordinary light-microscopic methods show up very clearly under the anoptral. It is astounding to note, however, how much the early pioneers in this field did manage to observe with their far simpler optical apparatus. It must be admitted, in fact, that little if any fundamental information on this subject has emerged since the works of Bizzozero (4), Eberth and Schimmelbusch (5) and Dekhuyzen (6), at least not on the morphological aspect. If we compare the hand-drawn figures of these early authors with the dark-field micro-photographs in the quite recent monograph of Fonio and Schwen-

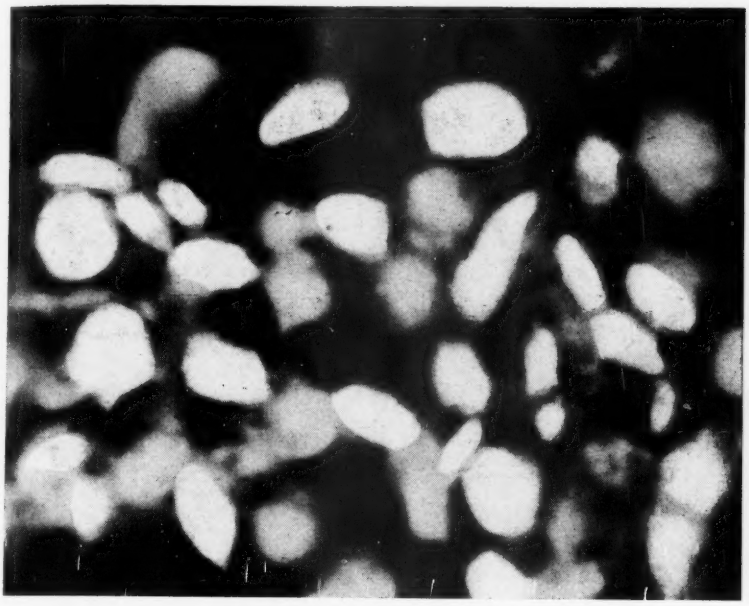

Fig. 1. — A group of thrombocytes in a fresh sample of heparinized blood. Some of the platelets have not yet had time to turn their discs and to adhere to the glass surface. Objective: Reichert anoptral 100 ×. Magnification 3100.

dener (7), we have to admit that the contribution of the method used by the latter authors is not very substantial.

Any microscopic specimen of fresh blood shows quite a number of thrombocytes, either singly or in clusters of up to hundreds of platelets each. To diminish the disturbing effect of masses of erythrocytes, it is advisable to dilute the sample with plasma, preferably heparinized. If we are quick enough in getting the specimen onto the microscope stage, we may even obtain a side view of some platelets (Fig. 1). Very soon, however, all the platelets turn their discs and adhere to the glass surface or to their neighbour platelets. This tendency to adhere was already known to the above-mentioned early investigators. In 1886, Eberth and Schimmelbusch described the »viscous metamorphosis» of platelets, which, according to them, was the cause underlying the formation of thrombi — a process that is not connected with the coagulation of blood. Ordinary white thrombi, they stated, are composed almost solely of thrombocytes and do not contain fibrin.

The views of Eberth and Schimmelbusch were strongly attacked in 1904 by Bürker (8), who found that the use of coagulation-inhibiting substances inhibited platelet destruction also. (Of the various substances tested by Bürker, the leech extract is the only one which could be termed an anticoagulant today.) After quantitative calculations, Bürker even considered the possibility of the thrombocytes acting as a source of fibrin.

Our observations confirm the old views of Eberth and Schimmelbusch. The addition of an anticoagulant (heparin, citrate) to the blood sample causes no change in the activities of the thrombocytes, although fibrin threads do not appear. If no anticoagulant is used, the threads form in the well-known way, at some places »radiating» from thrombocyte clusters. This radiation is never evident enough to be taken as a proof of the allegedly great part played by the platelets in blood coagulation, as it has been presented in many textbooks since the appearance of Bürker's publication. If we examine the specimen dispassionately, we find innumerable multiple crossings in the fibrin network which might equally well be regarded as centers of radiation, although they contain no thrombocytes. On the other hand, individual platelets and even groups of them can be seen frequently divorced from all contact with the fibrin network. Still, we do not feel justified from our observations in going so far as to disclaim the coagulation-promoting effect of thrombocytes altogether. Such an effect may exist, but it probably has no local character.

The most beautiful specimens of living thrombocytes can be obtained in the following way. A drop of blood is allowed to remain on a slide for 20—30 sec. During this time, a great number of platelets will adhere to the glass. After this, the blood drop with its major corpuscular elements is washed away with heparinized or citrated plasma. The thrombocytes which have attached themselves to the surface will remain there, unaffected by this manipulation. After furnishing the specimen with a coverglass and sealing the borders with melted paraffin, the behaviour of the platelets can be examined without disturbance from other blood constituents.

A remarkable feature of the thrombocytes is that after they have adhered to the surface they start spreading along it. This phenomenon also occurs on celluloid, cellophane and plexiglass (lucite). These surfaces are known to be more or less non-wettable

Fig. 2. — A group of thrombocytes photographed ½ to 8 min. after the specimen was made. The spreading can easily be seen. Shortly before the fourth picture was taken, a previously freelyfloating thrombocyte appeared from outside the field and attached itself to the upper edge of the group. In the last photographs we can see that even this newcomer has flattened out. Reichert anoptral 100 ×. Magnification 1650.

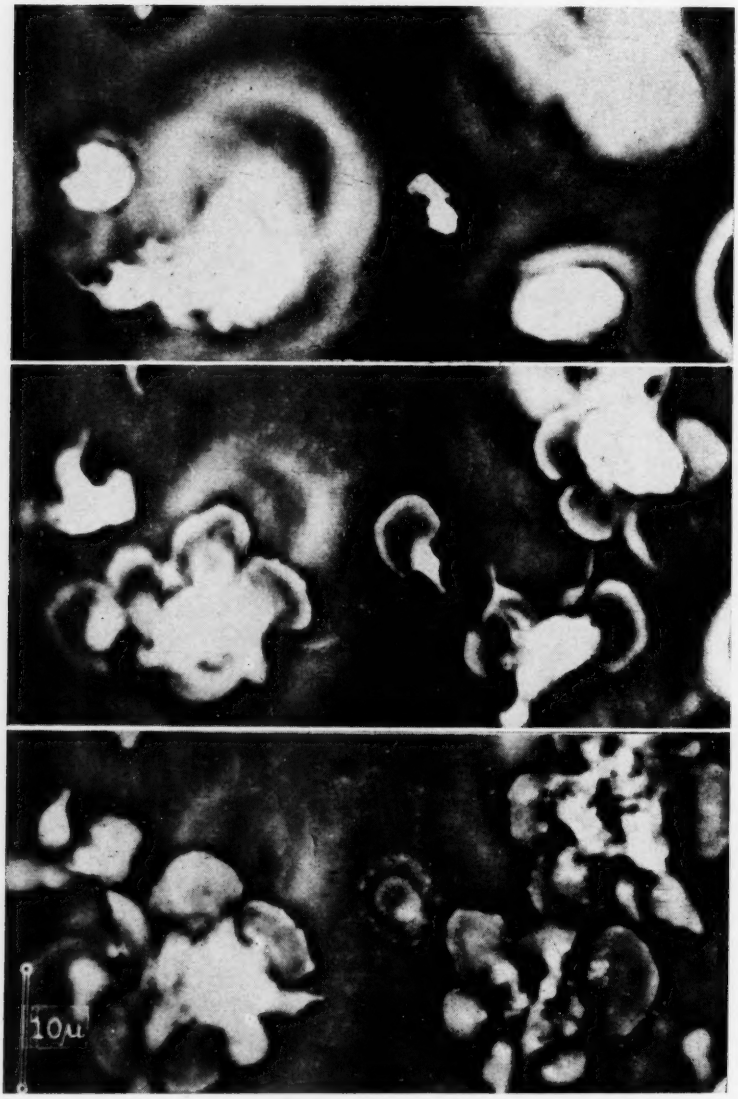

Fig. 3. — These serial photographs shows how whole agglomerates of thrombocytes adhere to the surface, at first loosely and then more firmly, until all the thrombocytes have flattened out. In the uppermost picture the still rounded-up groups are only partially in the focal plane. Time interval between the exposures about 3 min. Reichert anoptral 100 ×. Magnification 1580.

ones. The spreading property is, of course, appropriate to the main function of thrombocytes, *viz.*, to prevent leakage of blood through damaged capillaries and other vessels. The thrombocytes can be regarded as freely swimming living patches of the vascular bed. The authors have found that a thrombocyte is capable of spreading to a diameter of some 15 μ, *i.e.*, to cover an area more than 20 times larger than that occupied at the moment of its attachment. One could hardly imagine a more ingenious and efficient patch than one that not only adheres to the damaged spot but also spreads in all directions around it. Figs. 2 and 3 show this process in detail.

If we observe the behaviour of thrombocytes either in the clear spaces between rouleaux formations of erythrocytes in samples of whole blood or in specimens washed with plasma in the above-mentioned manner, we can see that the thrombocytes undergo a further series of changes during which hollow vesicles are formed. These changes appear whether or not the plasma is mixed with an anticoagulant. In agglomerates of thrombocytes, the vesicles appear 3—5 min. after the sample has been made. Within half an hour, the vesicles reach a size of 3—6 μ and protrude like balloons from the flattened individual thrombocytes or groups of them into the space between the object glass and the coverslip (Fig. 4). The vesicles appear dark in the anoptral image because they are less refractile, *i.e.*, more diluted, than the plasma. The authors cannot put forward any theory about the significance of these vesicles. It would be important to clarify the mechanism of their genesis and interesting to learn how the energy for filtering in thin fluid is obtained and how this fluid is kept free from the more refractile plasma constituents.

Let us now return to the spreading of the thrombocytes. We have observed that the presence of plasma colloids is not essential for this process. If the plasma is diluted more than 10 to 1 with Ringer or Tyrode solution, the spreading takes place in a normal way. With the use of a pure Ringer or Tyrode solution, the effect remains, although it is perhaps somewhat slower. In a solution containing four times the amount of solids of an ordinary Ringer or Tyrode solution, the platelets suffer no harm. The spreading starts normally as soon as the concentration is brought down to the normal level.

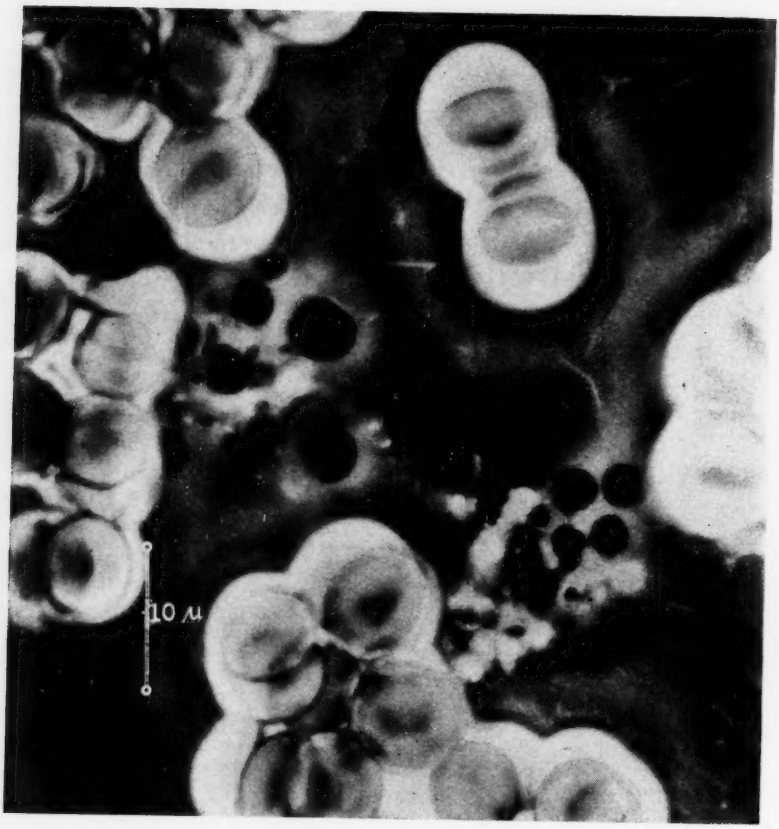

Fig. 4. — This picture shows the appearance of hollow vesicles around the thrombocyte groups in a blood specimen after about 30 min. Reichert anoptral 100 ×. Magnification 1920.

We have also made a few experiments with platelets by adding known amounts of various substances to the Tyrode solution. Cocain hydrochloride, 1 to 1,000, has no detrimental effect on the spreading, whereas a dilution of 1 to 100 inhibits both the adhesiveness and the membrane formation. Sublimate, 1 to 10,000, prevents the spreading and makes the thrombocytes resemble tiny thorn apples. Potassium benzylpenicillin, 1,000 I.U. per ml, has no effect on the thrombocytes.

When experimenting with several surface-active agents, we found that Tween 80 has a most conspicuous effect on the thrombo-

cytes. In a concentration of 2—3 per cent, it completely prevents the adherence and spreading effect described above. This is remarkable since surface-active agents generally have a wetting influence. On the other hand, many of the earlier statements on thrombocytes agree on one point: To prevent the platelets from adhering, the surface must be a non-wettable one. For preventing the coagulability of blood this may be true, but the Tween experiments certainly make the matter worth careful reconsideration as to the thrombocyte functions. We have also observed that thrombocytes which are already attached to the surface with broad membranous margins regain their original form very soon after they have been treated with Tyrode solution or plasma containing Tween 80. Tween 80 also causes agglomerations of platelets to disintegrate into single thrombocytes. If the Tween-containing solution is washed away with pure plasma or Tyrode solution, the thrombocytes regain their normal spreading properties.

If heparinized or citrated blood is passed through a column of fine quartz sand, the platelets stick to the sand particles. After this, the thrombocytes can be eluted with Tween-containing plasma or Tyrode solution (Fig. 5). A perhaps more practical way to obtain suspensions of thrombocytes is to add 2—3 per cent of Tween 80 to the blood sample and remove the erythrocytes and leukocytes by gentle centrifugation. The supernatant plasma will contain a great number of rounded-up thrombocytes which have no tendency to stick to one another. This suspension can be further concentrated by heavier centrifugation. However, the viability of thrombocytes in such suspensions containing Tween 80 is not very good. It is possible that other substances could be found that are less injurious to the thrombocytes than Tween 80. Of the other wetting agents that we have tried, Triton x—100, Teepol 32 and sodium and ammonium lauryl sulphate proved to be poisonous to the thrombocytes and to cause a rapid hemolysis.

This part of our work was concluded in the summer of 1953. Subsequently, the authors found that Minor and Burnett (9) used Tween 80 to reduce the irreversible agglomeration of platelets.

What is the factor that prevents the thrombocytes from sticking to the walls of the vascular bed? Without doubt, there must be some peculiarity in the innermost molecular groups of the vessel endothelium possibly combined with some special features in the blood,

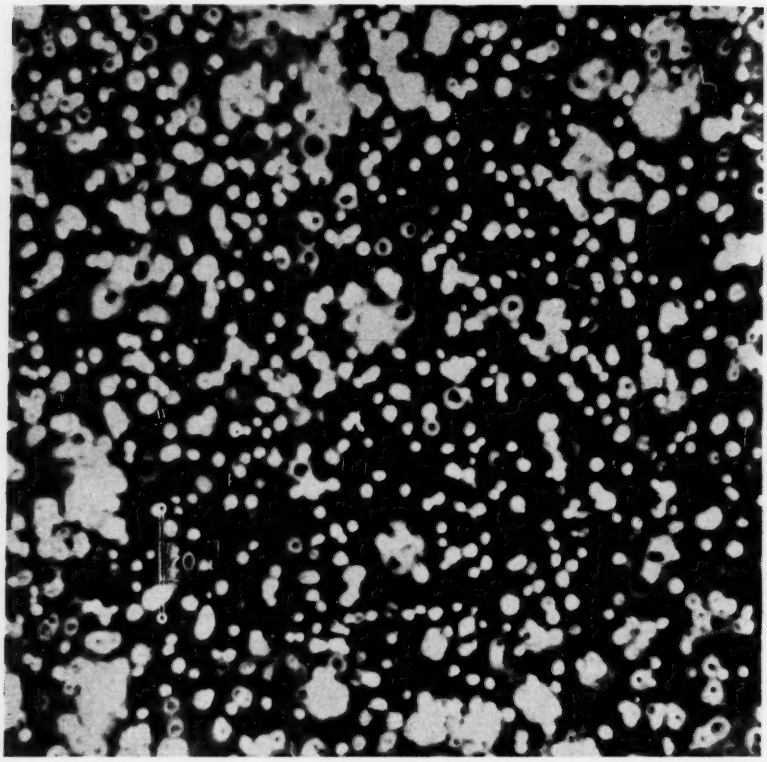

Fig. 5. — Thrombocytes eluted with a Tween-containing Tyrode solution from the quartz sand column into which they were absorbed from heparinized blood. Reichert anoptral 45 ×. Magnification 730.

which is responsible for this mutual repellence. We also know that the platelets do not stick to the larger blood corpuscles. They agglomerate easily, however, no matter how cautiously the blood sample is taken and how carefully it is stored in non-wettable dishes. In the circulating blood, the outermost molecular layer of the thrombocytes must, therefore, consist of some very labile groups which repel each other as long as they remain undisturbed or unaltered. Once the surface is altered, this layer becomes sticky, but it can be made repellent again by the application of Tween 80. For this reason, we may even assume that there are surface-active factors in the blood which keep the corpuscular elements, especially the thrombocytes, from adhering to each other and to the walls of

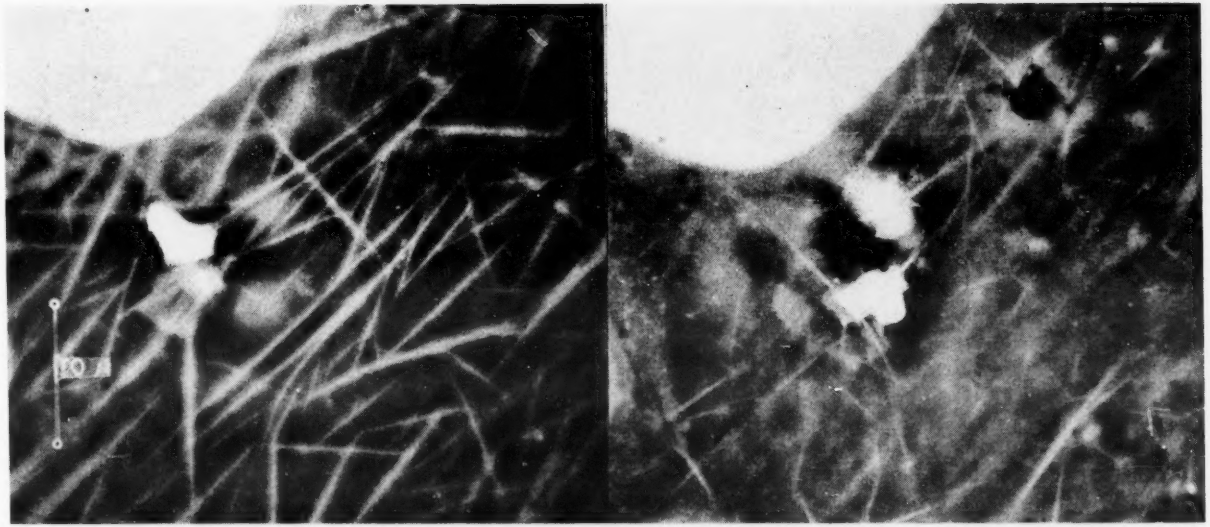

Fig. 6. — A drop of blood was spread between an object »glass» made of lucite (plexiglass) and an ordinary coverglass. The picture to the left was taken with the focal plane coinciding with the lower surface of the coverglass. A couple of thrombocytes and a dense fibrin network are seen. In the right-hand picture, the lucite surface in exactly the same place has been brought into focus. The spreading of thrombocytes takes place in a normal way, although the amount of fibrin is considerably less here than it is in the neighbourhood of the glass. In the upper right part of the picture is a hollow vesicle. Reichert anoptral 100×. Magnification 1890.

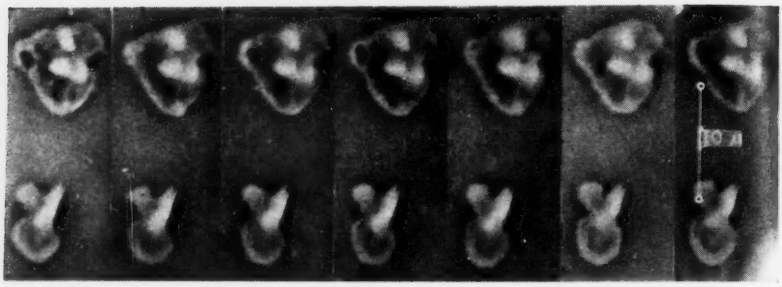

Fig. 7. — This serial picture, taken at ½ to 1 min. intervals, shows two flattened thrombocytes, the upper of which has a perforation in its margin on the left. The perforation becomes smaller until it disappears, but it soon opens up again. This process may be repeated several times and could be misinterpreted as being due to a contractile vacuole. Reichert anoptral 100 ×. Magnification 1500.

the system, unless need arises. If blood is extravasated, these agents become rapidly inactive.

Of other observations, we may mention that a short Tween 80 treatment for the purification of thrombocytes does not abolish vesicle formation (as illustrated in Fig. 4).

We have already mentioned that the quality of the artificial surface has but little to do with the spreading of thrombocytes. If plexiglass (lucite) is used instead of an ordinary object glass, the thrombocytes attach to it and spread in the normal way, although the fibrin formation is stronger close to the surface of the (ordinary) coverglass (Fig. 6).

Bessis and his co-workers (10) observed »contractile vacuoles» in the thrombocytes of man, frog and guinea pig. We have also made a few observations of effects resembling these, but our conclusions are slightly different (Fig. 7).

The thrombocytes of the frog, although large and nucleated, behave exactly the same as their human counterparts in all conditions, including that of treatment with Tween 80.

DISCUSSION

Many things concerning thrombocytes are still obscure. The genesis of platelets from megakaryocytes, although probable, has not been absolutely proved. The rate of turnover and the fate of thrombocytes are largely unknown. In view of the spreading pro-

perties of the thrombocytes, we may assume that the majority are sooner or later incorporated as integral parts of vascular walls, especially those of the capillaries. Thus, there is a continuous stream of matter from the bone marrow (if the thrombocytes emerge from it) to the vessel walls. This stream is preferentially directed toward those places in which the innermost layers of the walls are altered. In an inflamed part of the body, this vascular incorporation of thrombocytes may reach much higher values than in a normal part. Since there is still much confusion about the character of the changes in vascular thrombosis, we cannot draw further conclusions until the differences between the mean types of thrombi — white and red — have been finally clarified as to both their genesis and their structure.

SUMMARY

The authors present some results of their observations on living thrombocytes under the anoptral contrast microscope. Thrombocytes cannot be regarded as local factors in blood fibrination. The »viscous metamorphosis» of thrombocytes, described by Eberth and Schimmelbusch, consists of the flattening of thrombocytes along a surface, a process which is largely independent of the quality of the surface. During this process, the surface area of the thrombocytes may increase more than 20 times. Whole clusters of thrombocytes may flatten *in toto*. Hollow vesicles containing thin fluid appear around the flattened thrombocytes. The flattening takes place also in saline solution. Saline solutions concentrated four-fold do not abolish this property. Of many surface-active agents, Tween 80 in 2 per cent solution prevents the clumping and spreading of thrombocytes, dissolves existing clumps and detaches thrombocytes that have adhered to surfaces, without causing hemolysis. The contractile vacuoles reported by Bessis are probably rhythmically opening and closing perforations of the thrombocyte membrane. The authors stress the importance of clarifying the problem of different types of thrombi and present a hypothesis of a Tween-like although extremely labile constituent in the blood which normally prevents the adherence of thrombocytes.

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THE BLOOD HISTAMINE CONTENT IN HYPEREMESIS GRAVIDARUM

by

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The rise in the activity of histaminase, the histamine destructing enzyme of the blood, is characteristic of the human pregnancy beginning from the sixth or seventh pregnancy week. During the fifth month of pregnancy it reaches a level, which is about 1000 times as high as that of non-pregnancy, often even higher, and which remains rather unchanged until the delivery, decreasing sharply to the non-pregnant level after the removal of the placenta (for ref. see 1, 15). It must be assumed on the basis of the biological order that the rise in the blood histaminase activity is of physiological significance. Histaminase is not a specific histamine destructing enzyme, but histamine is physiologically and pharmacologically the most active substance among its substrates. The other substrates, such as putrescine and cadaverine are capable to potentiate the pharmacological effects of histamine by competing effectively with it for the common enzyme (23, 13). The physiological role played by histamine in the human pregnancy has been studied with interest since the discovery of the simplified method for the determination of the blood histamine content (2). The results have, however, been controversial. Some investigators are of the opinion that the histamine content of the blood (7, 11) as well as of the urine (17, 7) is increased during pregnancy, while the others consider it to be on the low level close to the normal (4), or even below

the normal a few hours before and after the delivery (12). Wicksell (21) noted no changes in the plasma or urine histamine content during parturition.

Very few histamine investigations have been performed on patients with hyperemesis gravidarum. Kapeller-Adler (11) isolated a considerable amount of crystalline histamine (on an average 1.82 mg/24 hr) from the urine of a woman with severe hyperemesis gravidarum, whereas no detectable quantity of histamine could be found in the urine of a normal pregnant woman by the same method.

Recent investigations (for ref. see 8, 9, 10) suggest temporary hypopituitarism followed by the secondary hypofunction of the adrenal cortex in hyperemesis gravidarum. Since it has been pointed out that the functions of the anterior pituitary and adrenal glands influence the histaminase content of the tissues and the histamine metabolism in animal experiments (for ref. see 6), it seemed to be justified to study the histamine metabolism in patients with hyperemesis gravidarum. The present work reports results of the blood and plasma histamine determinations in these patients. As the normal individual variations in the blood histamine content are rather large (for ref. see 3), an attempt was made to explain whether or not the disappearance of the syndrome causes any changes in the blood and plasma histamine content in these patients.

SERIES OF CASES AND THE METHOD

The histamine content of the blood and blood plasma was determined in 15 hospitalized patients with hyperemesis gravidarum. The age of the patients, the parity, the duration of pregnancy, and the beginning of vomiting from the first day of the last menstruation are recorded in the accompanying Table 1. In addition, the histamine content was determined in a pregnant woman who was hospitalized for severe ptyalism.

The blood samples for the histamine determinations were firstly taken on the following morning after the admission, before the patients had had any special treatment and, secondly, after the therapy when the symptoms had disappeared. The histamine determinations were performed at intervals of four to 20 days, the average interval being seven days. The blood plasma histamine

content only was determined in ten normal pregnant women, who were hospitalized for examination during the first trimester (in March, 1953). The histamine determination was made according to Code's (2) method, and in some cases the histaminase activity of the blood plasma was determined by using Wicksell's (20) method. Heparin was used to prevent the blood coagulation.

RESULTS

Table 1 shows the results of the blood and plasma histamine determinations in 15 patients with hyperemesis gravidarum. The total blood histamine content ranged between 2.0 and 21.0 μg% before the therapy, being on an average 6.6 μg%. The plasma histamine content ranged from 0.5 to 19.4 μ%, averaging 4.6 μg%.

After the therapy when the patients had stopped vomiting, the blood histamine content ranged from 1.5 to 12.0 μg%, being on

TABLE 1

Case Report No.	Age yrs	Parity	Time from the 1st Day of the Last Menses Days	Occurrence of Vomiting since the 1st Day of Last Menses Days	Before Treatment			After Treatment		
					Histamine Content		Histaminase Activity	Histamine Content		Histami nase Activity
					of Blood μg%	of Plasma μg%	of Plasma μg/ml/hr	of Blood μg%	of Plasma μg%	of Plasma μg/ml/hr
2128/51	23	I	102	50	9.6	9.0	0.25	2.4	1.4	0.60
2152/51	40	I	70	27	10.0	5.4	0.11	6.9	3.9	0.13
2205/51	30	I	90	43	8.4	8.2	0.90	5.0	2.3	—
2350/51	27	III	49	34	8.7	3.0	—	4.5	1.4	—
2129/53	39	II	50	36	2.0	0.5	—	2.2	0.5	—
2373/53	20	I	54	33	4.0	1.5	—	2.2	0.6	—
2842/53	30	I	110	65	3.4	2.4	—	3.0	2.8	—
2889/53	25	II	63	43	2.9	2.3	—	4.8	2.1	—
3543/53	36	I	57	40	2.5	2.0	—	1.5	0.9	—
3176/53	29	I	69	44	2.5	1.5	0.30	3.2	1.4	—
3247/53	33	II	74	30	5.0	2.0	—	5.5	5.4	—
3437/53	32	II	68	32	3.0	2.0	—	4.5	3.5	—
3499/53	32	III	118	30	3.5	2.1	—	2.6	0.6	—
3506/53	25	II	55	43	13.0	7.0	—	3.5	3.2	—
3834/53	32	I	67	35	21.0	19.4	—	12.0	10.9	—
Mean	30		73	39	6.6	4.6		4.3	2.7	

an average 4.3 μg%. The variation ranges for the plasma values were 0.5—10.9 μg% (mean: 2.7 μg%). The total blood and plasma histamine content decreased during the therapy 34.8 and 37.2 per cent, respectively.

In ten normal pregnant women, the plasma histamine content ranged from 1.4 to 6.5 μg% (mean: 3.4 μg%) during the first trimester of pregnancy.

In four hyperemesis patients the plasma histaminase activity ranged between 0.11 and 0.90 μg/ml/hr on the admission to the hospital. At the discharge, it was 0.13—0.60 μg/ml/hr in two convalescents.

A pregnant woman suffering from severe ptyalism showed no response to the treatment with atropine, progesterone, estrogen, and Percorten. Antrenyl, on the contrary, gave rather good results, and after the therapy the blood and plasma histamine values of the patient were 3.8 and 1.1 μg%, respectively.

THE THERAPY

Nine hyperemesis patients were injected 10,000—25,000 units of estradiol benzoate intramuscularly daily for three to six days. Five patients were administered saline infusions, Benadon, B-Complex and Percorten injections. A patient was treated with ACTH; 20 units of Cibacthen gel daily for four days. Prior to the therapy, Thorn's test (25 units of ACTH) was performed on the four patients, who were administered estrogen. As a matter of fact, these patients were thus treated with combined ACTH and estrogen therapy on the first day of the treatment. All the patients recovered rather quickly in spite of the varying treatment. Two patients, who recovered after the estrogen therapy, had relapses which, however, soon disappeared after the administration of saline infusions and Benadon injections. The results of the ACTH therapy in hyperemesis gravidarum have been reported earlier (9) and the comparative investigations of the various treatments will be discussed later in another connexion.

DISCUSSION

The results revealed that the normal pregnant women had during the first trimester a rather stabile plasma histamine level ranging between 1.4 and 6.5 μg%. Our results corresponded to those obtained

by other authors. The normal plasma histamine level ranges between 2 and 10 μg% in non-pregnant women and men (*cf.* 3, 18). Four out of the 15 hyperemesis patients had higher plasma histamine level on the admission to the hospital than the normal pregnant women. The level was close to the lower normal limit in ten patients, in three of them it proved to be extremely low (0.5—1.5 μg%). In spite of this, the average plasma histamine content was 35.3 per cent higher in the hyperemesis patients than in normal pregnancy, on an average. The total blood histamine level was in six cases higher in hyperemesis patients than in normal pregnant women according to Effkemann and Werle (4). In the hyperemesis patients the variations in the blood histamine level were approximately as large as those of the plasma histamine level. The most significant difference between the hyperemesis and normal pregnancy series seems to be the considerably greater variations in the plasma histamine values of the hyperemesis series as compared with the rather stabile histamine level in the normal pregnancy. No correlations were noted between the severity grade or relapsing tendency and between the blood or plasma histamine content.

Both the blood and plasma histamine content tended to decrease after the vomiting period. In nine cases out of 15 the blood and plasma histamine content fell more than 20 per cent, which can be held the error limit of the method. In one case only both the blood and plasma histamine content increased during the disappearance of the symptoms. The rise was not even then as convincing as in those cases, in which a rather high blood histamine level fell into a half, or even to a quarter of the pre-treatment value. The average histamine fall of the blood was about 35 and that of the plasma about 37 per cent during the treatment. The plasma histamine fell even below the average plasma histamine level of the normal pregnant women.

In the hyperemesis patients, the plasma histaminase activity was clearly increased above the non-pregnancy level (0.005 μg/ml/hr, *cf.* 1) after 69—102 days since the first day of the last menstruation. No correlation could be noted between the plasma histamine level and histaminase activity.

It is difficult to interpret the patho-physiological significance of the blood or plasma histamine values. Very little is known about the physiological role played by histamine and the regulation of

the plasma histamine level. The histamine level of the tissues and plasma is probably a resultant of the endogenous histamine formation and its elimination. The histamine level of an organism seems to be dependent on the functions of pituitary-adrenocortical axis and possibly of vegetative nervous system, and of the adrenal medulla. The intensity of the responses of the tissues to histamine depends, however, not only on the histamine concentration but also on the sensitivity of the tissues to histamine. The sensitivity, in turn, is dependent on the pituitary-adrenocortical and pituitary-thyroid systems, and probably also on even higher centers. In this respect, the adrenal cortex and thyroid seem to have antagonistic effects. On the basis of the experiments conducted on adrenalectomized animals, it is likely that also the hypofunction of the adrenal cortex can increase the histamine level of the tissues by decreasing the elimination and, in addition, increase the sensitivity to histamine. The thyroid hyperfunction, a general phenomenon in adrenocortical insufficiency, increases the hypersensitivity to toxic agents.

It has not been noted, however, that histamine is directly related to hyperemesis syndrome or vomiting symptoms in general. The exogenous histamine induces, in small doses, headache and other phenomena based on vascular responses in normal individuals. Vomiting is one of the severe symptoms of human histamine intoxication. The observations (19) that histamine inhibits serum and nerve cholinesterase suggest indirect effects. It has to be kept in mind in the general evaluation of the physiological role played by histamine during the pregnancy that histamine may have local intrinsic effects (Dale), which may be different from the extrinsic effects of histamine.

Hyperemesis gravidarum has been considered an allergic reaction to the secretion of the corpus luteum (5). Youssef and Barsoum (22) were of the opinion that the gonadotrophic hormone acts as an allergen. On the other side, hyperemesis patients show no typical allergic manifestations. Conversely, bronchial asthma often recovers during the pregnancy. The hyperemesis syndrome can hardly be considered a typical allergic reaction, although the patients with hyperemesis show signs of hypersensitivity. It has not been pointed out that some allergic reaction would cause liberation of histamine in these patients.

Though this discussion suggests a non-allergic dysadaptation in hyperemesis gravidarum, there may also be a possibility that the hyperemesis syndrome and allergy have characteristics common to both of them, perhaps even some common basic features. Insufficiency in the systems maintaining the homeostasis of the organism could be considered such. The deficiency to maintain the homeostasis becomes apparent in both of these states, *e.g.*, in the histamine metabolism. In normal pregnancy, the organism is capable to maintain the homeostasis from the beginning with regard to the histamine level of the plasma and probably of the whole organism. This power seems to be defective in hyperemesis patients. These patients show larger variations in the blood histamine content and it is claimed that they excrete larger amounts of histamine into the urine than do the normal pregnant women (11). Larger variations in the blood histamine content have also been noted in allergic subjects than in normal individuals (16, 18), and enormous amounts of histamine have been observed to be excreted into the urine by asthmatic patients (14). The reason for the large variations in the blood histamine level may be the endogenous histamine formation (or liberation), larger than normal, or the decreased enzymatic destruction, or both of them together. The histamine metabolism in hyperemesis gravidarum needs further investigations, which may be directed most profitably to the study of the histamine urinary excretion by using the new methods that have been developed recently.

SUMMARY

The blood and plasma histamine content was studied in 15 patients with hyperemesis gravidarum on the admission to the hospital and after the disappearance of the symptoms. Before the therapy the values for the blood and plasma histamine ranged between 2.0—21.0 μg% and 0.5—19.4 μg%, respectively (means: 6.6 μg% and 4.6 μg%, respectively).

On an average after seven days, when nausea and vomiting had recovered, the histamine content of the blood was 1.5—12.0 μg% (mean: 4.3 μg%) and that of the plasma 0.5—10.9 μg% (mean: 2.7 μg%), the decrease being 34.8 and 37.2 per cent, respectively.

In the group of ten normal pregnant women the plasma histamine level ranged between 1.4 and 6.5 μg% (mean: 3.4 μg%).

The plasma histaminase activity ranged from 0.11 to 0.90 μg/ml/hr in four hyperemesis patients on the admission to the hospital. It showed no relationship to the plasma histamine values.

The patho-physiological significance of the unusually large variations in the blood histamine level in hyperemesis gravidarum is discussed and the results are compared with similar observations made in allergy.

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FROM THE MEDICAL CLINIC, THE DEPARTMENT OF BACTERIOLOGY AND SEROLOGY, AND THE SURGICAL CLINIC, UNIVERSITY OF TURKU

SEPTICAEMIA AND DERMONECROSES ASSOCIATED WITH MICROCOCCUS PYOGENES VAR.AUREUS

A CASE SUCCESSFULLY TREATED WITH ERYTHROMYCIN AND SKIN GRAFTS

by

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(Received for publication December 3, 1954)

Strains of *Micrococcus pyogenes var. aureus* are known to produce exotoxins (1) which have a haemolytic and leukolytic action on rabbit blood, effect tissue necrosis and cause death of the animal. Enterotoxins released by certain strains also produce lesions in the human intestine (1). In 1901, Neisser and Wechsberg (6) observed that skin necrosis developed when they immunized rabbits with cultures of *Micrococcus pyogenes var. aureus* strains. These authors assumed that the necrosis was caused by haemolysin or leukocidin. According to Parker (7), there also exists a special dermonecrotic toxin. It is today generally assumed that α-haemolysin might be identical with the lethal, necrotizing exotoxin (1, 13).

Thal and Egner (10) studied experimentally the anatomico-pathological changes that result from local injection of micrococcal toxins. In summary, they reported that the pathological changes indicated an infarction of the skin. Prolonged segmental spasm of small arteries and veins was produced when micrococcal toxin was infiltrated around them. Capillary permeability was altered in such a way that stagnation of blood resulted. It was postulated that these two mechanisms acting together were responsible for the local dermal ischemia and subsequent necrosis.

Skin gangrenes are evidently very rarely caused by *Micrococcus pyogenes var. aureus* in man. Several cases of facial (2, 3, 5) and vulval gangrenes (4) as well as disseminated skin gangrenes (8) have, however, been described in which the only micro-organism isolated was *Micrococcus pyogenes var. aureus*. In a paper dealing with micrococci resistant to antibiotics, Spink (9) described a case in which a resistant micrococcal strain produced extensive skin gangrene and which ended fatally. A culture of the same strain was found to effect extensive skin necrosis in a rabbit.

In February, 1954, a patient with extensive skin necrosis which was later traced to a *Micrococcus pyogenes var. aureus* strain producing dermonecrotic toxin was admitted to the Medical Clinic of the University of Turku. Since several interesting features were exhibited by the case, and since the patient responded favourably to treatment, the course of the disease is described in detail.

CASE REPORT

The patient was a 44-year-old unmarried woman. All her close relatives were in good health. The gynaecological anamnesis was normal. There was no history of venereal disease. Appendicectomy had been performed at the age of 28; otherwise, she had been in good health up to 1950. From Dec. 16 to 19, 1950, the patient had been treated at the Medical Clinic of the University of Turku for tuberculous pleural effusion (septicaemia?) (Record No. 3701/50). On admission, the patient had a high fever and a rash. These symptoms disappeared completely in 10 days following administration of antibiotics (penicillin, chloramphenicol). She then recovered from the pleurisy in the normal manner. Acid-fast bacilli were cultured from the pleural fluid. The patient was transferred to a tuberculosis sanatorium for after-treatment. It may be mentioned that a penicillin-resistant *Micrococcus pyogenes var. aureus* strain was cultured from the patient's throat at this stage.

The patient was subsequently in excellent health, until she became ill of sore throat and fever at the end of January, 1954. The fever did not subside, and soon after black patches appeared on her forehead. *She had not taken sulpha, antibiotics or other drugs.* On Feb. 7, the patches suddenly expanded and also appeared on the arms and legs. A marked sensation of heat was felt by the patient in these regions.

The patient was admitted to the Medical Clinic on Feb. 19 (Record No. 732/54). It was established that the skin on the forehead, cheeks, the dorsal parts of the forearms, the gluteal region and the dorsal regions of the thighs and legs was necrosed and black. The patient's general condition was relatively good, although elevation of temperature (*ad* 39°C) persisted. The

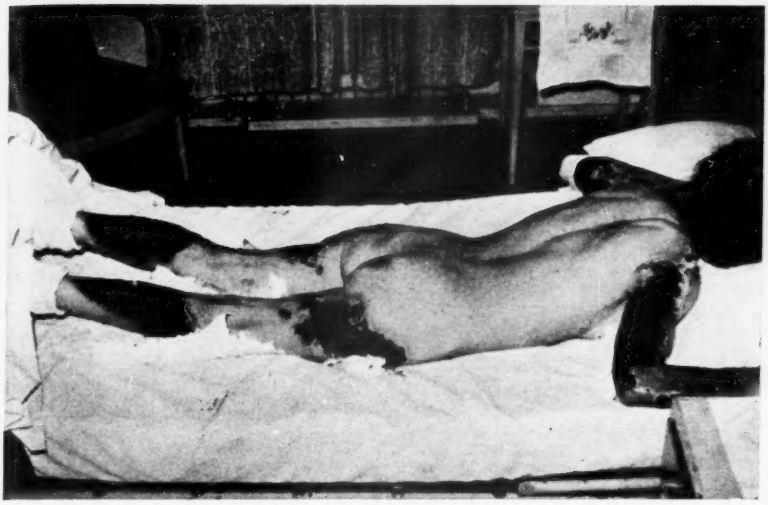

Fig. 1.

Fig. 2.

erythrocyte sedimentation rate was 90—125 mm/hr.; the blood picture was anaemic, and a relative leukopenia (2,400—3,000 leukocytes/cu. mm) was evident. X-ray examination of the thorax revealed extensive pleural thickening on the right side, but no fresh parenchymatous changes were evident. The non-protein nitrogen was 16 mg per 100 ml of serum. The

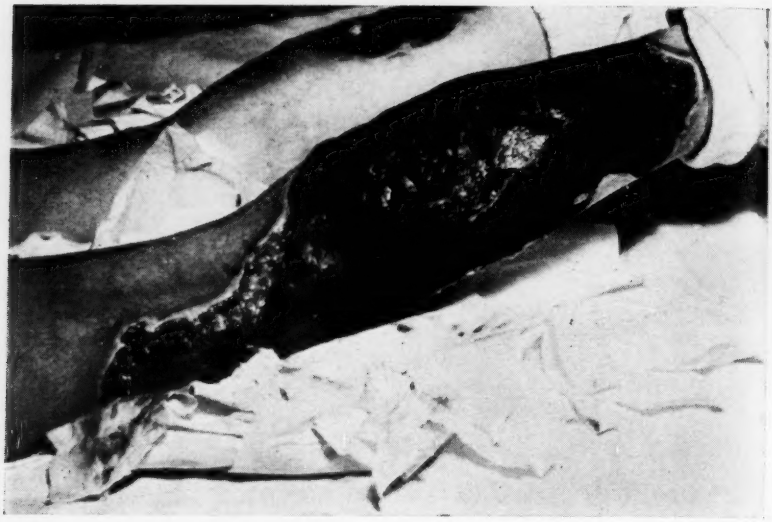

Fig. 3.

coagulation of blood was found to be normal. »L. E.» cell tests were negative (on four occasions). Six blood cultures taken during the three days after admission were all negative. On the black areas large blisters developed within a few days. Haemorrhagic fluid taken from a blister contained only a few erythrocytes and granulocytes. Gram staining and bacterial culture gave negative results.

Massive doses of antibiotics (penicillin, streptomycin, oxytetracycline, both successively and concurrently) were administered, and the patient was also given sulpha (Supronal). In addition, she received cortisone (300 mg initial dose, 100 mg maintenance dose) for two weeks. The patient's temperature elevation persisted (*ad* 39° C). The necrosis did not spread, but the necrosed regions gradually became quite hard (Fig. 1, 2 and 3). Since the bacterial culture of the blister fluid was negative, the observed changes were not suspected to be of bacterial origin.

On Feb. 24, a specimen was taken from the margin of the necrosed region. Pathological examination of the specimen revealed that the epithelium was absent from a large area. In its place was a necrotic ulcer, the base of which was covered with purulent granulous tissue. The pathologist (Helminen) concluded that the changes were of the pemphigus type, although blister formation was no longer evident at this stage. The skin change had possibly originated in a septic condition.

At the same time also, new blood cultures were made and a specimen for bacterial culture was taken from the necrosed area. The specimen contained a large number of polymorphonucleate leukocytes and several clusters of Gram-positive cocci. *Micrococcus pyogenes var. aureus* was found in the blood

Fig. 4.

Fig. 5.

and pus. A Sabouraud culture was negative. According to sensitivity tests, the strain was very sensitive only to erythromycin.

Administration of erythromycin (1,200 mg daily, total dose 42 g)was, therefore, started at once. The patient's condition soon improved, and the temperature rapidly fell to the level of 37—38° C. The necrosed skin areas also began to heal, and the red colour disappeared from the surrounding areas. The extensive sphacelus could be gradually removed (Fig. 4). The necrosed areas of the forehead and gluteal regions underwent spontaneous epithelization. The thighs, legs and upper arms, however, remained covered by open granulous surfaces. Owing to this, the patient was transferred to the Surgical Clinic on May 7, and Thiersch free skin grafts were transplanted twice. The transplantation succeeded satisfactorily. On Aug. 23, the patient returned for a follow-up examination. Her general condition was good. The erythrocyte sedimentation rate was 4 mm/hr. and the haemoglobin concentration 15.1 g per 100 ml. The skin had remained intact in the grafted regions (Fig. 5). The joints were freely mobile.

When the presence of micrococcal infection was established, both the antistaphylolysin (ASTA) and antistreptolysin (AST) titres were followed. The upper limits of »normal variation» employed in our methods are 1.5 for ASTA and 200 for AST (11). Since the results of serological tests are greatly dependent on the conditions prevailing at the time of their performance, the serum specimens from the patient were frozen (—20° C) and titrations were performed in parallel. The following results were obtained:

Date	ASTA	AST
1/3	9	400
3/3	9	
9/3	18	200
16/3	36	200
23/3	50	200
30/3	18	280
9/4	25	
17/4	25	100
24/4	25	125
3/5	36	200
22/5	36	100
22/6	36	100
24/8	18	90

It will be seen that the antistaphylolysin titres increased greatly, while the antistreptolysin titres remained within normal limits. An electrophoretic analysis of the serum conducted during cortisone treatment (Feb. 22) showed that the albumin content was less than normal, whereas the total globulin content had increased, especially the γ-fraction (relative percentages: albumin + α_1 : 35.0, α_2 : 11.3, β : 14.6 and γ : 38.8). A second analysis was performed on March 17, when the ASTA titre (36) was extremely high. Compared with the preceding results, the percentage of α_2-

globulin had increased, as also had that of the albumin + α_1 (relative percentages: albumin + α_1: 45.2, α_2 : 17.8, β : 14.8 and γ : 22.1).

BACTERIOLOGICAL PROPERTIES OF THE ISOLATED STRAIN

The *Micrococcus pyogenes var. aureus* strain effected extensive haemolysis on sheep blood agar, gave positive coagulase and mannite reactions and produced yellowish pigment. Attempts were made to produce toxins from the strain. The strain was cultured according to Walbum (12) in meat extract broth containing 0.5 per cent peptone (Alwitt Trading Co., Ltd., London), 0.03 per cent $MgSO_4$ and 0.2 per cent Na_2HPO_4. The culture was grown five days in an atmosphere of 20—25 per cent CO_2. After this period, the medium was centrifuged for 15 min. at 15,000 rpm. The supernatant liquid was used as toxin. This toxin was diluted with saline solution for purposes of titration. Even in a dilution of 1 to 10,000 the toxin haemolyzed rabbit red cells during one hour's incubation at 37° C, and a dilution of 1 to 100 produced skin necrosis in a rabbit in three days. These phenomena are obviously due to an α-haemolysin and a dermonecrotic toxin. A rabbit given an injection of 1 cc per kg of body weight of cell-free toxin died 30 hours later. The ability to produce fibrinolase was tested in broth agar with the addition of 12 per cent plasma (13). A marked clearing of the agar around the colonies was observed which may indicate the formation of fibrinolase.

The strain was resistant to sulphathiazole, sulphamezathine, penicillin, streptomycin, chlortetracycline and oxytetracycline. It showed a slight sensitivity to chloramphenicol, neomycin and magnamycin. It was moderately sensitive to 2 per cent Amisept (quaternary ammonium salt, Lääke Oy) and to Supronal. The strain was, however, very sensitive to erythromycin.

COMMENT

In summary, in the case presented, a 44-year-old woman had a condition characterized by a fever and sore throat, which was followed by extensive necrosis of the skin on the face, limbs and gluteal regions, which areas were initially covered by a haemorrhagic bulla. Blood and bulla fluid cultures were at first negative. Biopsy of the skin lesion proved it to be purulent. A bacterial culture from the necrosed area and a repeat blood culture both yielded a coagulase-

positive haemolytic *Micrococcus pyogenes var. aureus* strain that produced necrosis in the skin of a rabbit, was resistant to most antibiotics and was highly sensitive only to erythromycin. Administration of the latter brought about a marked improvement in the patient's condition. Two skin grafts were performed on the patient, both of which were successful. She recovered satisfactorily, although initially about 14 per cent of her skin area was necrosed.

The condition obviously was a septicaemia, possibly due to a strain of *Micrococcus pyogenes var. aureus* producing a dermonecrotic toxin. This conclusion is supported by the rise observed in the serum antistaphylolysin titre. Judging from the results obtained in the electrophoretic analyses, the antistaphylolysin appears to be associated to some extent with the α_2 or α_1 fractions. The fact that the first bacterial culture made from the bulla was negative suggests that the skin lesion was initially of toxic origin, and that the bacterial invasion was a secondary occurrence. It seems certain that the bacteriological examination and sensitivity determinations were of decisive significance for the patient's recovery.

SUMMARY

The authors describe an unusual case of septicaemia associated with a strain of *Micrococcus pyogenes var. aureus* that produced a dermonecrotic toxin. The strain in question was found to be resistant to almost all common antibiotics. Erythromycin was an exception in this respect both *in vitro* and *in vivo*. Although nearly 14 per cent of the patient's skin was necrosed, she recovered after administration of erythromycin and skin transplantations.

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FROM THE CHILDREN'S CLINIC, UNIVERSITY OF HELSINKI, AND FROM THE RINNE-HOME, INSTITUTION FOR MENTAL DEFECTIVES OF THE DEACONESSES' HOSPITAL, HELSINKI

INFLUENCE OF SUPERINFECTION ON THE TUBERCULIN SENSITIVITY OF BCG-VACCINATED PERSONS ISOLATED IN INSTITUTIONS

by

OLE WASZ-HÖCKERT and MÄRTA DONNER

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The role of superinfection is an ever-fresh subject of discussion in medical journals. The »Ergebnisse der gesamten Tuberkuloseforschung» of Jan. 1954, for instance, devotes a whole chapter to the matter (18). The different aspects of the problem are still unsolved.

We should like to report some observations in order to contribute to the knowledge of:

a) the influence of superinfection on the tuberculin sensitivity of BCG vaccinated persons,

b) the possibility of distinguishing between pure vaccination allergy and superinfection allergy in BCG vaccinated persons.

SURVEY OF THE LITERATURE

It has long been known that superinfection changes tuberculin sensitivity in spontaneously tuberculin-positive persons. Meinicke (7) and Much (8) have already reported an increase in the sensitivity of the staff of tuberculosis hospitals. They assume the cause to be inevitable superinfection in the course of their work. Redeker (10) has seen a considerable increase in sensitivity, tested according to Pirquet, especially in new, untrained T.B. hospital staff members.

Rich (11) and Wallgren (13) also mention that superinfection increases tuberculin sensitivity in spontaneously tuberculin-positive persons.

On examining the tuberculin sensitivity of BCG vaccinated persons in order to discover the extent to which the sensitivity had disappeared there was found a clear difference between individuals exposed to the risk of tuberculosis and those living in a tuberculosis-free milieu (3, 12, 14). The number of tuberculin-negative persons was much smaller in the first group. This seems to indicate that superinfection keeps the vaccinated persons tuberculin positive. However, directly contradictory results have been obtained in a similar investigation (1).

About 1930 Heimbeck (5) observed a difference between BCG allergy and tuberculous allergy. The tuberculin reactions of the former he thought to be pale, weak, with little infiltration. Later Hertzberg observed the strength of the tuberculin sensitivity in 13,101 BCG vaccinated persons in Oslo over a period of 4—5 years. With 85% the sensitivity diminished or was persistently constant. In 14.5% the sensitivity first diminished or persisted at the same level and later suddenly became stronger, remaining at the new level for the rest of the observation period. Hertzberg thougth the latter group to be superinfected. The diameter of the Mantoux-reaction (1.0 mg) increased at least 40 mm in one-third of the cases and 20—40 mm in the remaining two-thirds. According to him, pure vaccination allergy can thus be distinguished from superinfection allergy (6).

Vuilleumier (19) obtained similar results. He observed the tuberculin sensitivity in the pupils of a nurses' training school after BCG vaccination. During the observation period which was from 2 to 69 months (average 30 months) about one-fifth of the pupils became tuberculin negative afresh and in three-fifths of them the sensitivity remained constant. In one-fifth the reactions strengthened. This increase he thought to be caused by superinfection. In addition he assumed that at least part of those who showed no change in the strength of the reaction were superinfected. This assumption he based on the fact that a high number of the non-vaccinated, tuberculin-negative pupils turned positive during their training.

Myren (9), who examined 534 inhabitants of a rural community one to two years after BCG vaccination, found strengthened

reactions in 30 to 50% of them. This number varied with the amount of vaccine given. Half of the 56 persons who received 2.0 mg showed an increased reaction. The others had received 0.1—0.2 mg. He concluded that an increase in tuberculin sensitivity is common among the vaccinated and that only in a small part of the individuals can it be attributed to superinfection.

OWN OBSERVATIONS

Since 1949 we have followed the course of the tuberculin sensitivity after BCG vaccination in children and adolescents in institutions for mental defectives. Both the inmates and the staff of these institutions have been carefully observed for the occurrence of tuberculosis; they can be considered to have lived in a milieu quite free from the risk of tuberculous infection. The results of these investigations have been described by us previously (16, 17). The strength of the tuberculin sensitivity caused by BCG vaccination was followed up to four years after the vaccination. These figures are used here for comparison. The mongoloids are now excluded as their tuberculin sensitivity has been shown to be stronger than that of other groups (4). The number of persons in the comparison series was thus 170 at the beginning of the experiments, 84 at the end.

Two fairly small, isolated wards had cases of open tuberculosis. Tuberculin tests of the vaccinated individuals in these wards were continued, though they were of course excluded from the above-mentioned series. Thus we have been able to observe the possible influence of superinfection on the tuberculin sensitivity.

Material. — Group I. The first of the above-mentioned two wards had eleven BCG vaccinated adolescents or young adults. Their age varied between 15 and 27 years. They had earlier been vaccinated after negative Mantoux-tests (1.0 mg) with 0.1 cc of BCG vaccine from the Sahlgren Hospital in Gothenburg and were all tuberculin positive three months after the vaccination. Three years later one of the spontaneously tuberculin-positive, non-vaccinated inmates of the same ward fell ill with pneumonia and was isolated ten days later when tuberculosis was suspected. Stomach lavage culture showed growth of tubercle bacilli. The patient died eight months later and the autopsy confirmed the diagnosis of widespread pulmonary tuberculosis.

Group II. The second ward had, among other patients, five BCG vaccinated children. Their age varied between 5 and 15 years. They had been vaccinated in the same way as Group I after negative Mantoux-tests and were also tuberculin positive three months afterwards. Eight to fourteen months after the vaccination they were exposed to tuberculous infection by another of the inmates, a 17-year old non-vaccinated girl with a naturally acquired tuberculin sensitivity. She suffered from repeated pneumonias and erythema nodosum during this period. She was isolated fourteen months after vaccination of the others and died three months later of pulmonary tuberculosis. Stomach lavage culture gave positive findings.

Thus Groups I and II were exposed to tuberculous infection by a young adult in the same ward. No one in the groups showed any signs of active tuberculosis subsequently.

Technique. — All the test persons were carefully tuberculin tested at intervals of a half to one year by one of us (M.D.). We used the Mantoux technique with various dilutions of Old Tuberculin from the Copenhagen State Serum Institute and doses of 1.0 mg, 0.1 mg, and 0.01 mg. The area of reddening and infiltration was

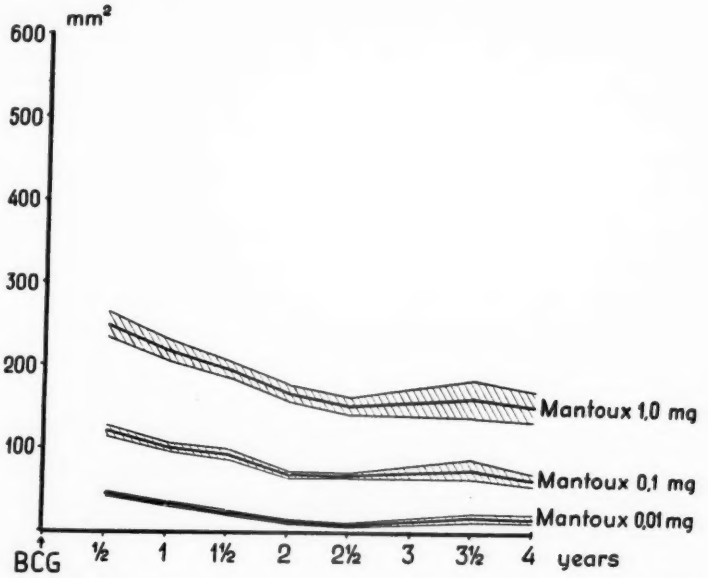


Fig. 1. — Diagram showing average strength of tuberculin reactions in BCG vaccinated not superinfected persons, tested with Mantoux 1.0 mg, 0.1 mg, and 0.01 mg. The shaded areas give the limits of the standard error.

estimated 72 hours later by measuring the longest diameter and the diameter at right angles to the longest. The strength of the reaction was given in sq mm as the product of these diameters.

Results. — In the comparison series of persons with pure BCG allergy the tuberculin sensitivity gradually diminished during the observation time. This is illustrated in Fig. 1.

Among the eleven inmates of Group I, who were exposed to superinfection three years after the vaccination, there was an increase in the mean tuberculin sensitivity after this time. This is illustrated in Fig. 2.

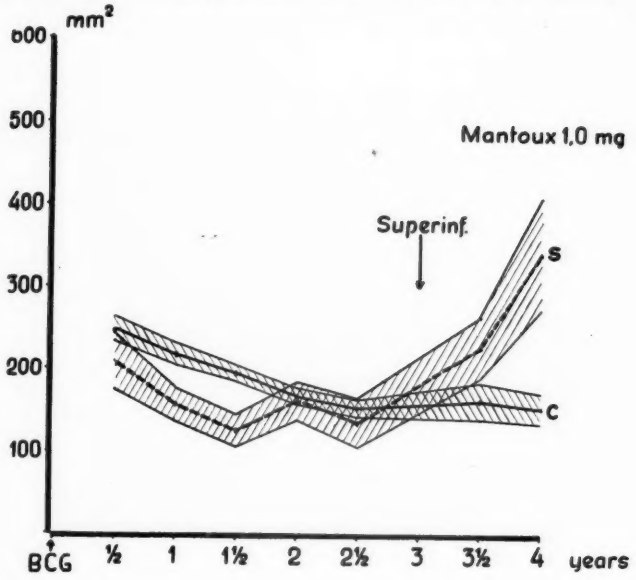
A similar increase was seen among the five vaccinated children in Group II, exposed to infection within approximately one year of vaccination. As this group could be followed for a longer period after the exposure we were able to obtain a picture of the progress of tuberculin sensitivity of persons exposed to superinfection.

The results of the examinations in Group I and II agree: the tuberculin sensitivity increases after the exposure to tuberculous infection. This increase seems to culminate one to one and a half years later and then the sensitivity weakens again.

Analysis of the individual tuberculin reactions showed that only five of the eleven in Group I and three of the five in Group II differed clearly in tuberculin sensitivity from the control series. They alone revealed increased reactions. It is easily understood that when calculating the means of the reactions of all the members of the groups the dispersion will be great as only this part of them have strengthened reactions. It follows from this that statistical calculation of the standard error of the difference between the means of the tuberculin reactions of the exposed persons on the one hand and the controls on the other does not give reliable results. Anyway there seems to be a significant difference for Group I one year and for Group II half a year, one year and one and a half years after the superinfection compared with the controls.[1]

The tuberculin reactions in these five and three patients with a definitely changed tuberculin sensitivity are thus much stronger than the means for all the members of the groups. They fall approximately within the upper limit of the standard error or are still stronger, and probably give a truer picture of the increase in tuberculin sensitivity after superinfection. We have in any case no evidence

[1] The calculations were made by E. Kaila, Ph.D.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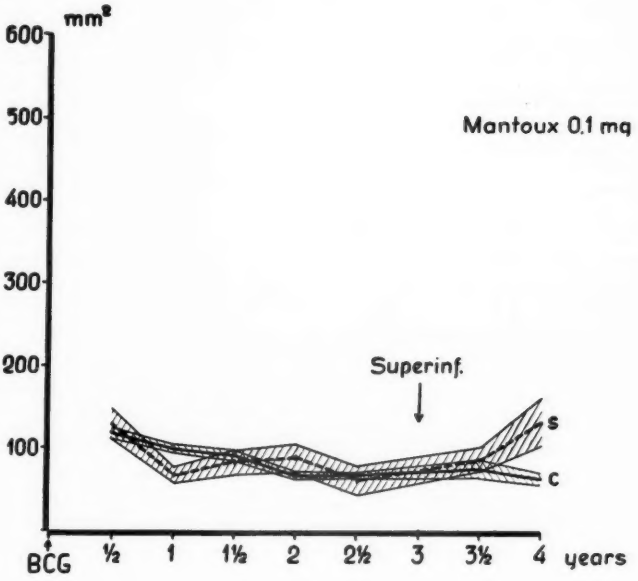
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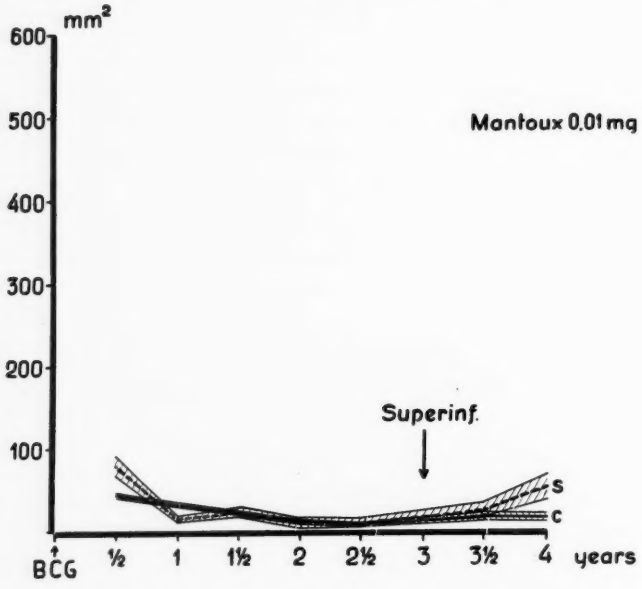


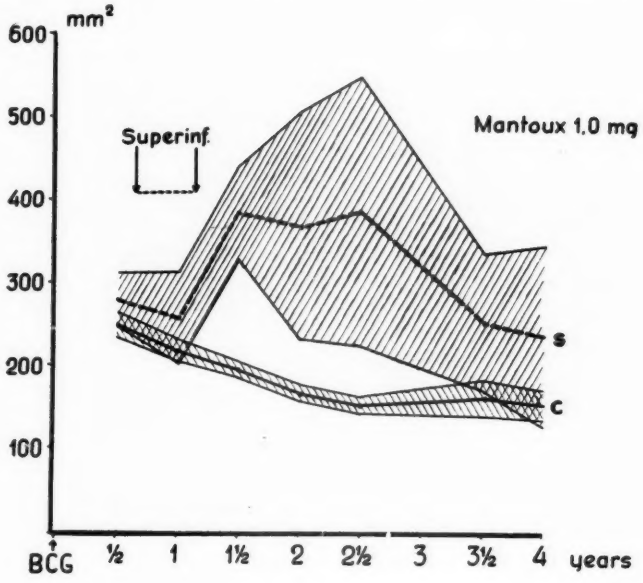
Fig. 2. — Diagram showing average strength of the tuberculin reaction in 11 BCG vaccinated persons (S) exposed to tuberculous superinfection three years after vaccination, compared with non-exposed BCG vaccinated controls (C). The shaded areas give the limits of the standard error.

that it was these and not the other six or two inmates who were superinfected. It is, however, certain that part of those who showed a real reaction in Group I were in closer contact with the tuberculous patient than those who did not react. In fact three of them slept in the same room as the T.B. patient. The chances of contact are great however, in daytime too as all the inmates of the ward use the same day- and dining rooms.

DISCUSSION

We have observed an undoubted increase in the tuberculin sensitivity of both the groups of BCG vaccinated persons exposed to tuberculous superinfection. In the group which was followed up for three years after this exposure to infection the sensitivity was all the time stronger than in the controls though it also gradually decreased.

The results can be compared with the opinion of Rich (11) and Wallgren (13) regarding the change in tuberculin sensitivity after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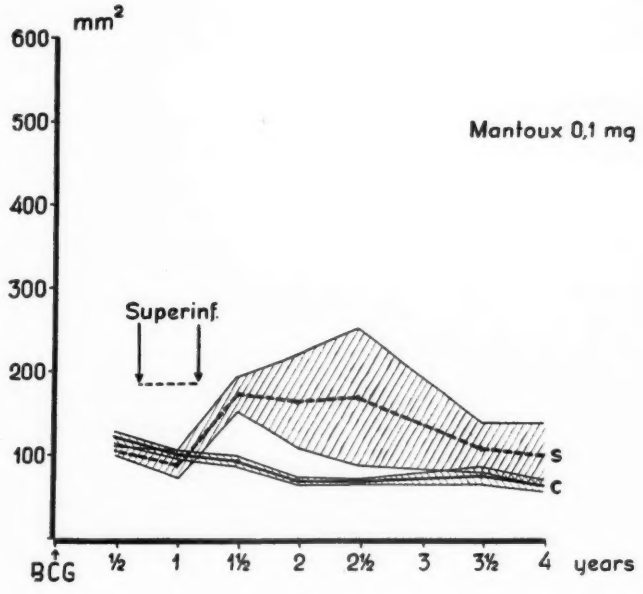
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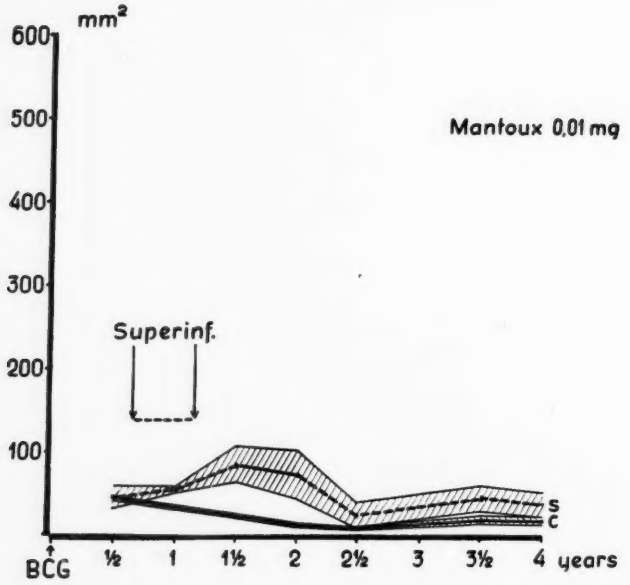

c

Fig. 3. — Diagram showing average strength of the tuberculin reactions of five BCG vaccinated persons exposed to tuberculous infection 8 to 14 months after vaccination (S), compared with non-exposed BCG vaccinated controls (C). The shaded areas give the limits of the standard error.

superinfection in spontaneously tuberculin-positive persons. They also confirm the results obtained by several authors (3, 12, 14) who have shown that superinfection keeps BCG vaccinated persons from becoming negative again. They agree with the results of Hertzberg (6) that superinfection causes an increase in the strength of the reactions of BCG vaccinated persons.

However, our investigation series and the course of the investigation differ from those of the above-mentioned authors. The series consists of persons isolated in institutions, where tuberculosis can be said to be under control. The test subjects are tested at regular intervals and the time of the superinfection is known. Therefore our observations, theoretically, ought to illustrate well the question of the influence of superinfection on tuberculin sensitivity.

We also wish to stress the purely practical aspects of our observations. It seems to be possible for the physician to decide whether the tuberculin positivity in a BCG vaccinated individual is caused by the vaccination only or if the vaccinated subject has also

got a virulent tuberculous infection in addition. This was stressed earlier by Heimbeck (5), Wasz-Höckert (14), Hertzberg (6), and Anttila (2). In the Childrens Clinic, University of Helsinki, quantitative Mantoux-tests have been used for several years on the initiative of Wasz-Höckert and found of benefit in the differential diagnosis of etiologically uncertain lung cases (15).

SUMMARY

Tuberculin sensitivity after BCG vaccination was observed for four years among individuals isolated in institutions. During this observation period eleven BCG vaccinated inmates of one ward and five of another were exposed to tuberculous infection by two other inmates, young adults. The tuberculin sensitivity of these two groups was followed for one and three years, respectively, after the superinfection. A considerable increase in the strength of the reactions was seen, and a significant difference between the sensitivity in the exposed persons and those who had lived in a tuberculosis-free milieu.

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FROM THE DERMATOLOGICAL CLINIC, UNIVERSITY OF HELSINKI

TREATMENT OF HERPES ZOSTER NEURALGIA WITH MASSIVE DOSES OF VITAMIN B_{12}

by

JUHA HELLE and KYLLIKKI OHELA

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There is at present no specific treatment for herpes zoster, though there is scarcely a drug or therapy that has not been tried out. Varying results have recently been obtained with broad-spectrum antibiotics, cortisone, and ACTH. Since the symptoms of the disease, herpetic lesions and neuralgic pain, disappear rapidly even without therapy, it is difficult to assess the efficiency of the treatment used. In this connection Sulzberger and Wolf (12) pointed out: »The proof of any measure in zoster depends on evidence that it is capable of rapidly curing or relieving a convincingly large number of severe, intractable and long-lasting postzoster neuralgias. As yet we have not remedy which succesfully meets these criterias.»

Since good results have been obtained with massive doses of vitamin B_{12} both in the neurological manifestation of pernicious anaemia and in certain other neuropathies such as neuritides caused by alcohol and diabetes (9, 10, 11) and trigeminal neuralgia (2), we decided to experiment with massive doses of vitamin B_{12} as a therapy for the zoster pain.

MATERIAL AND METHODS

The series consisted of 47 patients with zoster pain, treated in the Dermatological Clinic of the University of Helsinki in the beginning of 1953. Six of them were hospitalized, the others were

outpatients. Nineteen patients were treated with vitamin B_{12} intramuscularly and indifferent local therapy. The dosage was 1000 micrograms of B_{12} daily for six days and thereafter every other day four times, *i.e.* ten times altogether.

In the control group 19 patients received local therapy only and 9 patients were given 150 mg of thiamin hydrochloride instead of B_{12}.

Table 1 shows the sex and age distribution of the patients in the different treatment groups.

TABLE 1

DISTRIBUTION OF ZOSTER PATIENTS ACCORDING TO AGE, SEX AND TREATMENT

Treatment Group	Male	Female	Total	Age in Years	
				Range	Average Age
Vitamin B_{12} and local therapy..	7	12	19[1]	23—82	52
Local therapy only	10	9	19	19—73	47
Thiamin hydrochlorid and local therapy	6	3	9[2]	21—69	48
Total	23	24	47		

[1] in 5 patients zoster occured in the area of n. ophtalmicus.
[2] in 2 » » » » » » » » »

Vitamin B_{12} therapy was started 1—11 days (on an average four days) after the onset of pain. Four patients who had had pain for 30, 30, 50 and 105 days constituted an exception in this respect. The patients who were given local therapy only attended the hospital 1—14 days (average five days) after the manifestation of pain; one of these patients, however, had suffered from pain for 56 days. Thiamin hydrochloride treatment was started 2—17 days (on an average five days) after the onset except with two patients who had had pain for 30 and 90 days.

RESULTS

The control period was long enough to permit an assessment of the therapeutical results for 40 patients out of the material of 47 patients (Table 2). 32 patients were followed-up for over a year.

In nine patients the pain continued at the termination of the control period, in one for two months and in eight for over a year

TABLE 2

RESULTS IN THE DIFFERENT TREATMENT GROUPS OF ZOSTER PATIENTS

	Number of Patients			Total No. of Patients
	Vit. B_{12} and Local Therapy	Local Therapy only	Thiamin Hydrochloride and LocalTherapy	
Pain ceased during or after treatment	13	11	7	31
Pain continued at the end of the control period..	4	3	2	9
End results could not be estimated because of too short control period ..	2	5	0	7
Total	19	19	9	47

TABLE 3

DURATION OF PAIN IN THE DIFFERENT GROUPS OF ZOSTER PATIENTS WHOSE PAIN CEASED DURING OR AFTER TREATMENT

Treatment Group	Total Duration of Pain		Duration of Pain since the Beginning of the Treatment		Number of Patients
	(Days)	Average	(Days)	Average	
Vitamin B_{12} and local therapy	2—44	14	1—17	6	13
Local therapy only	1—35	13	1—35	8	11
Thiamin hydrochloride and local therapy	2—17	11	1—13	6	7
				Total	31

after the initiation of treatment. These patients were equally divided among the therapeutical groups.

The termination of the pain in the treatment groups is set out in Table 3. It will be seen that in patients treated with vitamin B_{12} the pain ceased an average of six days after the beginning of the treatment and lasted altogether an average of 14 days. The corresponding figures for patients receiving local treatment only were 8 and 13 days and for patients treated with thiamin hydrochloride 6 and 11 days. Essential differences in the duration of the pain can

TABLE 4

PATIENTS WITH POST-ZOSTER PAIN AT THE END OF THE CONTROL PERIOD

Treatment Group	Ages of Patients (Years)	Duration of Pain before Treatment (Days)	Control Period (Months)
Vitamin B_{12} and local therapy	57	11	17.5
	65	50	13
	68[1]	6	16.5
	79	105	17
Local therapy only	43[1]	10	18
	44	56	13
	64	1	2
Thiamin hydrochloride and local therapy	54	90	13
	61	30	13

[1] Herpes zoster occurred in the area of n. ophtalmicus.

consenquently not be established in the different therapeutical groups.

The nine patients whose pain continued at the end of the control period are introduced in Table 4. The table shows their age, the duration of pain prior to the initiation of treatment and the lenght of the control period. Here again there is no difference between the therapeutical groups.

DISCUSSION

There are reports from many quarters of favourable results with the use of vitamin B_{12} in the treatment of herpes zoster patients. The first attempts of this kind date back to 1946, when Dickie (1) treated ten herpes zoster patients with liver extract with good results. Later Gaskell (3) reported 20 patients whose pain disappeared after one or two intramuscular injections of 4 ml of liver extract. Jolles (6) 1950 administered two ml of concentrated vitamin B_{12} daily for three days to 6 patients. Marked clinical and objective improvement followed upon the first injection and by the end of the first week all local signs had disappeared. Heyblon (5) reported the treatment of 11 cases; 8 recovered in two to six days. He recommended the use of 30 micrograms daily for eight days. 7 of the 11 patients in Grasso's (4) series showed marked improvement

after four to six days' treatment with 30 micrograms of vitamin B_{12} daily. Lövei and Antalóczy (8) described good results in 14 out of 15 cases with daily doses of 60 micrograms. Pain disappeared on about the third day of the treatment. Leitch (7) treated five zoster patients with massive doses of vitamin B_{12}. In each case the pain subsided or ceased entirely within twenty-four hours of the initial administration of 1000 micrograms of B_{12}. Retrogression and healing of the herpetic lesions began in 36 to 48 hours. Treatment was concluded four to eight days later, the dosage being gradually reduced. However Sancetta *et al.* (11) report a negative result with a zoster patient treated by them with 15 micrograms daily for a week.

Many investigators point out that their material is too small to permit definite conclusions. In the present material, also limited, we failed to establish any curtailment of the duration of zoster pain with vitamin B_{12} in comparison with the control groups. Patients to whom vitamin B_{12} had been administered spoke, however, of feeling in better general condition both during and after treatment. The patients of the control groups made no comparable observation. No harmful side effects were noted with the administration of massive doses of vitamin B_{12}.

SUMMARY

The effect of massive doses of vitamin B_{12} on zoster pain was studied in the Dermatological Clinic of the University of Helsinki. 40 of the 47 patients were followed-up for a sufficiently long period to permit an assessment of the treatment. 17 of them were treated with vitamin B_{12} and indifferent local therapy. 1000 micrograms of vitamin B_{12} was injected intramuscularly daily for six days and thereafter every other day four times. Of the 23 controls, 14 received local therapy only and nine were given thiamin hydrochloride instead of B_{12}. Vitamin B_{12} had no shortening effect on the zoster pain compared with the control groups.

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FROM THE MEDICAL CLINIC, UNIVERSITY OF TURKU

BROAD SPECTRUM ANTIBIOTICS

EFFECT ON THE LEVEL OF SERUM INDICAN AND ON THE URINARY INDICAN EXCRETION

by

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According to the prevailing opinion, indican is formed in the organism as a result of bacterial putrification in the intestine or elsewhere in the body. In the putrification, tryptophan is converted to indole, which then is oxydised to indoxyl and conjugated with sulfuric acid chiefly in the liver.

Certain observations, however, conflict with the foregoing opinion. One of them is the persistence of indicanuria also during continued fasting. This contradiction, however, is a seeming one only, as indole can be formed in the putrification of intestinal juice secreted during fasting. The increased excretion of indican in hepatic carcinoma reported in the literature [1] is more difficult to explain. Wooldridge *et al.* [2] sterilised the intestines of 6 test subjects with streptomycin and glucuoriolactone. Although both the aerobic and anaerobic cultures from feces became negative for all test persons, no decrease in the indican excretion was observed in 5 persons.

In the investigation reported in the following we studied the serum indican and the urinary indican excretion during a period when the test persons received certain broad spectrum antibiotics that strongly reduce the intestinal bacterial flora.

The serum indican and the 24-hour urinary indican excretion were first determined. The test subjects were then given the anti-

biotic under experiment for 6 days, during which the serum indican and the indican excretion were determined daily. Two subjects received neomycin and bacitracin *per os* in daily doses of 3 g of each antibiotic, 4 subjects received oxytetracycline, and 3 subjects chloramphenicol, also 3 g daily. The serum indican determination was made in the morning from fasting subjects by the Monias-Shapiro method and the urine indican by Parker's modification of Askenstedt's method. During the test period the subjects had the usual hospital food with a protein content of *c.* 100 g daily.

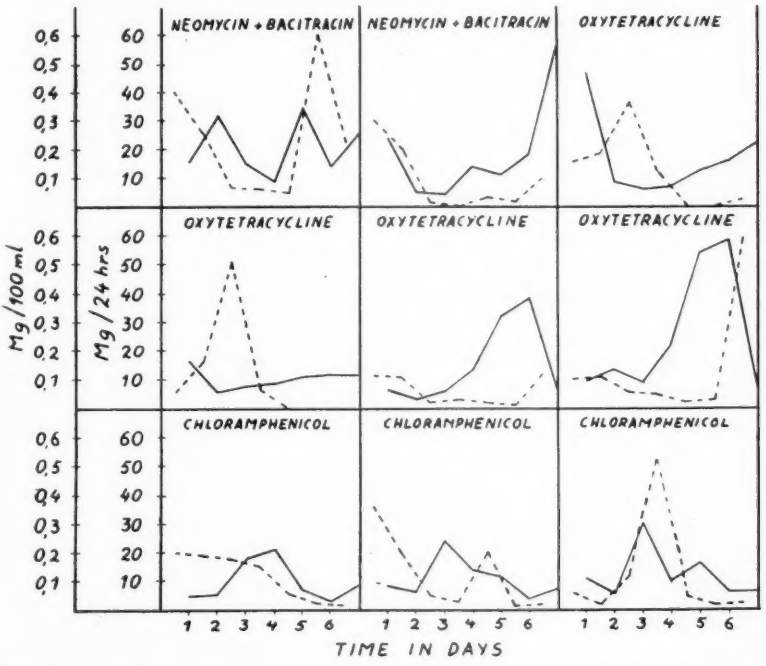

Fig. 1. — Diagram showing the effect of neomycin + bacitracin, oxytetracycline, and chloramphenicol on the level of serum indican and on the 24-hr urinary indican excretion. ——— serum level; — — — urinary excretion.

As seen from the illustration, the urinary indican excretion decreased markedly in all test persons during the administration of antibiotics; in some cases it disappeared completely. On the last test day an increase again occurred in some subject. In consideration of the fact that the antibiotics used are generally not capable of complete intestinal sterilisation, the results obtained in respect

to urinary excretion in this experiment are well in agreement with the opinion of intestinal origin of indican.

The effect of the antibiotics on the level of serum indican was considerably more indefinite and conflicting. During the administration of neomycin + bacitracin and chloramphenicol the serum indican followed to some degree the urinary indican excretion. although even considerable individual fluctuations in opposite directions were seen. In the experiments with oxytetracycline there was at first a tendency to a reduced serum indican level. This fall was followed in 3 cases by a definite and in 1 case by a slight rise, at the same time as the urine indican reached its lowest level or declined further. The changes seen are best accounted for by the assumption that oxytetracycline, contrary to the other antibiotics, influences not only the intestinal bacterial flora but also the renal indican excretion.

We may conclude from the results of these tests that the changes in the urinary indican excretion during the administration of antibiotics are evidence supporting the opinion indican is a product of intestinal putrification, whereas no definite conclusion in this respect can be drawn from the changes observed in the level of serum indican.

SUMMARY

Using 9 test subjects, the writers observed the level of serum indican and the urinary indican excretion during the administration of broad spectrum antibiotics. The antibiotics caused a decrease or complete discontinuation of urinary indican excretion in all cases whereas the changes seen in the serum indican level were conflicting.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI, AND FROM THE WIHURI RESEARCH INSTITUTE, HELSINKI

CORTISONE AND CIRCULATING ANTIBODIES

THE EFFECT OF A SINGLE DOSE OF CORTISONE ON THE CIRCULATING DIPHTHERIA ANTITOXIN AND THE NUMBER OF LYMPHOCYTES IN PREVIOUSLY IMMUNIZED RABBITS

by

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(Received for publication January 10, 1955)

In 1944—46 Dougherty et al. (3, 4, 5) reported that injection of adrenocorticotrophic or adrenal cortical hormones caused a transient enhancement of circulating antibody concentrations in previously immunized animals. The antibody concentration usually returned to the preinjection level within twenty four hours following administration of the hormones. A simultaneous transient reduction in the number of circulating lymphocytes called forth the conclusion that the increase of circulating antibody concentration might be due to a release of stored antibodies from the lymphocytes, undergoing a disintegration under the influence of cortisone.

In recent years, this subject has aroused the interest of a number of other investigators. Some of them have obtained results similar to those of Dougherty et al. (1, 2, 6, 10), whereas others, have not been able to confirm their findings (7, 8, 11, 13). Thus there seems to be a discrepancy between the conclusions reached, although variations in experimental conditions restrict the possibilities to compare the results obtained by different authors. In most studies semiquantitative, rather inaccurate serum dilution methods have

[1] Aided by a grant from the Sigrid Jusélius Foundation.

been employed for assay of antibody concentration (1, 2, 3, 4, 5, 10 etc.). In most instances, in which absolute quantitative precipitin methods have been employed, no significant change has been found in circulating antibody nitrogen after administration of adrenocorticotrophic or adrenal cortical hormones to previously immunized animals (7, 8, 11, 13). But these methods, applicable only when considerable amounts of the antibody are present, may leave small variations in the antibody concentration undetected.

An excellent antigen-antibody system for immunological studies is that consisting of diphtheria toxin and antitoxin. Even minute amounts of toxin or antitoxin can be accurately determined, independently of the quantitative precipitin reaction, by the rabbit or guinea pig intracutaneous test. The supreme sensitivity of the intracutaneous test is illustrated by the fact that even as little as 0.01 units/ml of antitoxin may be detected, whereas the quantitative precipitin method gives valid data only when at least 10—15 units/ml are present. As far as we know, this antigen-antibody system has not been previously employed in the study of the effect of adrenocorticotrophic or adrenal cortical hormones on antibody concentration in immunized animals.

In the work here reported, the effect of a single dose of cortisone on the circulating diphtheria antitoxin concentration of rabbits, previously immunized with diphtheria toxoid, was studied; simultaneously the number of circulating lymphocytes was noted.

MATERIALS AND METHODS

Diphtheria Toxoid. — A diphtheria toxoid preparation of 80 per cent purity, made by Lederle Laboratories Division (»Purogenated Diphtheria Toxoid No. 2929—169») and containing 1752 Lf per mg of protein nitrogen, was used for immunization of the rabbits.[1]

Immunization Procedure. — Six young male albino rabbits, weighing 1500—1900 g, were injected subcutaneously in the back every second day with 50 Lf of the diphtheria toxoid, contained in one ml of the diluent. A series of six injections was given, totalling 300 Lf. The immunization was started in October 1953. Samples for determination of the antitoxin concentration were taken by ear bleedings at varying intervals, up to October 1954. At this time, the second phase of the experiment, stimulation by cortisone, with subsequent determinations of circulating antitoxin and of number of lymphocytes, was carried out.

[1] Our sincere thanks are due to Lederle Laboratories Division, Pearl River, N.Y., for the Diphtheria toxoid.

Assay of Antitoxin. — The technique of the intracutaneous rabbit test was followed (9) with the exception that, for reasons of economy, guinea pigs were used instead of rabbits. In preliminary experiments, consisting of simultaneous assays of known antitoxins in rabbit and guinea pig skin, no significant differences in the results were obtained.

The standard antitoxin used was obtained from the Danish State Serum Institute (lots DI 38, 39, and 42). A comparison of this standard antitoxin (lot DI 38) with an American standard (lot A-7, The National Institute of Health) gave identical potency for both.

For all assays the same lot of diphtheria toxin was used. This toxin had been prepared by Dr. Melvin Cohn of the New York University Department of Microbiology. The MLD (Minimum Lethal Dose) of this toxin had been re-determined in 1952 by Dr. Merrill W. Chase of the Rockefeller Institute for Medical Research.[1]

In our hands, a minimal erythematous reaction in guinea pig skin was caused by injection of 0.1 ml of a mixture consisting of equal volumes of the toxin (diluted to contain 0.4 MLD per ml) and the standard antitoxin (diluted to contain 0.01 units per ml). Therefore, toxin diluted to contain 0.4 MLD per ml was mixed with equal volumes of dilutions of the serum to be assayed for its antitoxin content, and 0.1 ml of the mixture was injected into the guinea pig skin. A borate-saline buffer *p*H 7.4 was used as diluent for toxin and antitoxin. In the case of toxin, 200 *ug* of gelatin per ml had been added to the buffer in order to prevent surface denaturation.

Estimation of the Circulating Lymphocytes. — The total number of leucocytes per ml of blood was counted by the usual counting chamber technique. Slides for differential count were made from the same ear bleeding, and the percentage of lymphocytes was estimated. Thus the absolute number of circulating lymphocytes could be calculated.

Stimulation with Cortisone. — Fifty mg of cortisone acetate (»Adreson», Organon Inc.), contained in two ml of diluent, were injected subcutaneously into the back of the rabbits. Ear bleedings for assays of Antitoxin and the number of lymphocytes were taken immediately before, and 5, 9, 13, 23, and 80 hours following stimulation with cortisone. Four of the rabbits were thus stimulated with cortisone, whereas two animals served as controls, being given two ml of diluent instead of cortisone.

RESULTS

The antitoxin concentrations at different times following the immunization with toxoid are shown in table 1. The immunization schedule extended over a period of twelve days (300 Lf of toxoid in doses of 50 Lf every second day). From the table it can be seen

[1] We wish to thank Dr. Melvin Cohn for the diphtheria toxin, and Dr. Merrill W. Chase for the re-determination of its MLD.

TABLE 1

ANTITOXIN CONCENTRATIONS AT DIFFERENT TIMES FOLLOWING IMMUNIZATION WITH DIPHTHERIA TOXOID

Rabbit No.	1953					1954		
	Oct. 16 Pre-bleeding	Nov. 2	Nov. 12	Nov. 23	Dec. 14	Jan. 4	Sept. 19	Oct. 15
534	< 0.01	0.1—1.0	1.0 —5.0	0.01—0.1	0.01—1.0	> 0.01	0.01—1.0	0.05—0.1
535	< 0.01	1.0—5.0	0.01—0.1	0.01—1.0	0.01—1.0	> 0.01	0.01—0.1	0.05
536	< 0.01	1.0—4.0	0.01—0.1	0.01—1.0	0.01—1.0	> 0.01	0.01—0.1	0.05
537	< 0.01	0.1—0.3	1.0 —2.0	0.1 —1.0	0.1 —1.0	> 0.1	0.01—0.1	0.05—0.1
538	< 0.01	0.1—2.0	1.0 —5.0	0.01—1.0	0.01—1.0	> 0.01	0.01—0.1	0.01—0.05
540	< 0.01	1.0—3.0	1.0 —5.0	0.1 —1.0	0.1 —1.0	> 0.1	0.1 —1.0	0.1 —0.17

Immunization was started October 19.

The figures of the table give the antitoxin concentrations expressed in units pro ml of serum.

that the antitoxin concentrations reached their peak values in all instances within two weeks of the cessation of the immunization. In all cases the peak values remained below five units of antitoxin per ml of serum, at the same time exceeding one unit per ml of serum. At the end of an observation period of one year, all animals still had antitoxin in their circulation, although the concentration in all except one had gone down to 0.1 units per ml or less. The only exception was a concentration of 0.1—0.15 units per ml of serum. At this point the second phase of the investigation followed, consisting of stimulation of the animals by cortisone, with subsequent estimation of circulating antitoxin concentrations and of the number of lymphocytes at short intervals. Data from this experiment are shown in table 2.

The test bleedings were taken as follows:

Prebleeding	around	8.30	A.M.	on	October	15
5 hours bl.	»	1.30	P.M.	»	»	»
9 »	»	5.30	»	»	»	»
13 »	»	9.30	»	»	»	»
23 »	»	7.30	A.M.	»	»	16
80 »	»	4.00	P.M.	»	»	18

The rabbits had been fasting overnight before the experiment. They then were allowed to eat and drink from 9 to 12 A.M. From 12 to 10 P.M. no food, only water was given. From 10 P.M. on, the animals were allowed to eat and drink without restrictions.

TABLE 2

EFFECT OF CORTISONE ON THE ANTITOXIN CONCENTRATION AND THE NUMBER OF CIRCULATING LYMPHOCYTES

Rabbit No.	Time Relationship to Cortisone Stimulation	Antitoxin Concentration per ml of Serum	Number of Circulating Lymphocytes per ml of Serum	Percentage of Lymphocytes of the Total Number of Leucocytes	Total Number of Leucocytes per ml of Serum
	Before	0.05—0.1	5,700	54	10,500
	5 hours after	»	4,600	31	14,800
	9 » »	»	4,200	18	23,100
534	13 » »	»	4,600	14	25,800
	23 » »	»	6,600	26	25,500
	80 » »	»			
	Before	0.05	3,400	38	8,900
	5 hours after	»	2,500	22	11,300
	9 » »	0.05—0.1	2,900	17	17,400
535	13 » »	0.05	1,300	9	14,300
	23 » »	»	5,000	25	20,300
	80 » »	»			
	Before	0.05	1,300	12	11,200
	5 hours after	0.03—0.05	1,500	11	14,200
	9 » »	0.05	1,500	11	13,500
536	13 » »	»	3,900	21	18,800
	23 » »	»	3,200	18	17,800
	80 » »	»			
	Before	0.05—0.1	6,200	51	12,100
	5 hours after	»	3,800	21	13,700
	9 » »	»	2,700	17	16,100
537	13 » »	»	2,300	13	17,500
	23 » »	»	7,800	36	21,900
	80 » »	»			
	Before Saline	0.01—0.05	7,600	66	11,600
	5 hours after	»	8,100	61	13,200
538	9 » »	»	8,900	63	14,100
(control)	13 » »	»	7,400	69	10,700
	23 » »	»	7,400	60	12,500
	80 » »	»			
	Before Saline	0.1 —0.17	5,700	48	11,800
	5 hours after	0.17	5,700	53	10,800
540	9 » »	»	6,100	44	13,800
(control)	13 » »	»	9,000	58	15,500
	23 » »	0.1 —0.17	6,300	51	12,300
	80 » »	0.17			

The data presented in table 2 show that no change whatsoever in the concentration of circulating antitoxin was observable in test bleedings taken at intervals of 5, 9, 13, 23, and 80 hours following stimulation with cortisone, while the reducing effect of cortisone on the number of circulating lymphocytes was definite in three out of a total of four rabbits. The single rabbit (No. 536) not affected by cortisone in this respect exhibited an exceptionally low initial number of circulating lymphocytes. In all instances, the number of lymphocytes returned to the initial level or exceeded it within 23 hours following stimulation with cortisone. Because of the leucocytosis following cortisone administration, the relative number of lymphocytes, expressed as percentage of the total number of leucocytes, decreased considerably more than the absolute number of lymphocytes. In control rabbits, receiving saline instead of cortisone, no significant change in the number of lymphocytes was seen. The slight transient increase in the absolute number of lymphocytes was obviously associated with a normal fluctuation in the total number of leucocytes during the observation period. This correlationship is confirmed by the circumstance that the percentage of lymphocytes calculated from the total number of circulating leucocytes remained practically constant throughout the observation period.

DISCUSSION

In the present study, administration of a single dose of cortisone to rabbits stimulated a year previously with diphtheria toxoid did not cause any measurable change in the concentration of circulating antitoxin within an observation period extending to eighty hours. At the same time a definite biological activity of the cortisone dose administered was indicated by changes in the number of circulating leucocytes, particularly by the reduction in the number of circulating lymphocytes.

At the time of cortisone stimulation, the animals still had small amounts of antitoxin in their circulating blood. Considering the half-life of about thirty days of actively produced antibody (12), this circumstance must be regarded as an indication of a state of active antibody production, still going on at a diminishing rate one year after stimulation of the animals with the antigen.

An attempt was made to create a state where no measurable circulating antibody would be present any more. This state was nearly, but not completely reached, despite the relatively long observation period of one year. It was hoped that in such a state any cortisone effect upon the release of antibodies supposedly stored within the lymphocytes would have greater chances to become manifest, even in the case of a very minute effect of this kind. For the same reason and considering the fact that the accuracy of the antitoxin assay technique is optimal at low antitoxin levels requiring the least possible number of diluting procedures for the skin test, the rabbits were not hyperimmunized, as has been done in many of the earlier studies, including those by Dougherty et al. (3, 4, 5). We felt entitled to expect that the accuracy and sensitivity of the assay of antitoxin by skin test technique would possibly compensate for the lower total antibody concentration of the whole organism, including the supposed antibody depots within the lymphocytes.

Summarizing, it can be stated that the results obtained did not give any support to the hypothesis that cortisone causes a release of stored antibodies from lymphocytes undergoing a disintegration under the influence of cortisone.

SUMMARY

The effect of a single injection of cortisone on the concentration of circulating diphtheria antitoxin was studied in rabbits immunized a year previously with diphtheria toxoid. No change whatsoever was caused in the circulating antitoxin levels, despite the biological activity of the cortisone dose indicated by the reduction in the number of circulating lymphocytes.

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FROM THE DEPARTMENT OF BACTERIOLOGY AND SEROLOGY, UNIVERSITY OF TURKU

SEROLOGICAL IDENTIFICATION OF ESCHERICHIA STRAINS FROM URINARY INFECTIONS AND SUPPURATIVE CONDITIONS

by

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The various attempts made to identify different Escherichia strains by serological methods (2, 3, 6, 7, 12, 15, 16, 18, 19) were practically speaking unsuccessful until Kauffmann (9, 10) discovered a thermolabile antigen, the L antigen.

This L antigen prevents the agglutination of living and formalin-treated Escherichia strains in the homologous O immune serum, which finding provided an explanation for the previous discordant results. The L antigen is destroyed by heating one hour at 60° C. and by alcohol treatment, whereupon the strain is agglutinated by O serum. Many of the O inagglutinable strains were not agglutinated by O sera even after the heat treatment; these strains were designated A forms by Kauffmann. This property was later ascribed to a thermostabile A antigen (11). A two-hour period at 120° C. is required to destroy its power to prevent agglutination by O serum (21). Knipschildt (11) established the existence of a third antigen, the thermolabile B antigen. The latter differed from the L antigen in that its agglutinin-binding capacity was not destroyed by heating. The A, B and L antigens are usually referred to as the K antigens (8) and are known as the capsular (A) and envelope or surface (B and L) antigens. The thermolabile flagellar H antigen of Escherichia has been shown to be monophasic (8).

The present antigenic schema of Kauffmann-Knipschildt-Vahlne (8) comprises 25 O groups, which are further subdivided into several types according to their K and H antigens.

Vahlne (21) investigated 1414 E. coli strains isolated from normal faeces, 406 from urinary infections, 179 from cholecystitis. Forty-two per cent of the strains from normal faeces, 61 per cent from urinary infections, 44 per cent from cholecystitis belonged to the first 25 O groups. Parvis and Grosso (17) were able to type 28 per cent of 1048 strains from various sources. Of 68 strains isolated from urinary infections, they could classify 26%.

The purpose of the present work has been to investigate on the basis of the antigenic schema elaborated by Kauffmann-Knipschildt and Vahlne the serological distribution of Escherichia coli strains isolated from urinary infections and various suppurative processes.

MATERIAL AND METHODS

Of the 483 investigated strains, 359 were isolated at the Department of Bacteriology and Serology, University of Turku and 124 kindly supplied by the Department of Bacteriology and Serology, University of Helsinki, during the period from 1 January to 15 October, 1954. Four hundred and eight of the strains had been isolated from urinary infections, and 75 from various suppurative processes (19 from gall bladder infections and 56 from ear and nose infections and abscesses).

In order to obtain pure cultures of the strains, subcultures were performed twice from one single colony. The subcultured strains were then tested biochemically and serologically.

The following biochemical reactions were carried out: mannitol fermentation (acid and gas), indole production, hydrogen sulphide production, liquefaction of gelatin, urea decomposition, citrate utilisation, and Voges-Proskauer and methyl-red reactions. These reactions were studied as described before (4), except that the Voges-Proskauer reaction was performed according to the method of Barritt (1). The haemolytic power was tested by the technique of Kauffmann (8) using sheep red cells (22). The results were examined after 24 hours' incubation at 37°C.

The immune sera were prepared as described by Grönroos (4). In addition to O and K immune sera corresponding to the antigens of the diagnostic schema of Kauffmann-Knipschildt-Vahlne, immune serum for O 78 and sera prepared using Escherichia strains (111 : B4, 55 : B5, 26 : B6, 44 : K?, 86 : B7, E611 [1] and Canioni) isolated from cases of infantile diarrhoea were employed in the diagnosis of the strains. The H antigens were studied using immune sera H 1—33.

[1] According to Dr. Joan Taylor (20), this strain has been classified as 126 : B16 by Dr. F. Kauffmann.

The O antigens were determined preliminarily in 0.2 ml test tubes employing seven mixed O sera by a simplified technique (5). For this test, the broth cultures were heated at 100° C. for two hours. Since according to the diagnostic schema A antigens occur only in the O groups 8 and 9, broth cultures were also tested after heating at 120° C. for two hours with a mixed immune serum of O 8 and O 9. The further identification closely followed the directions of Kauffmann (8). When the strain under study was agglutinated in a dilution that was the same as, or deviated by only one dilution, from that of the control organism, it was considered to possess the antigen in question.

The routine determination of the K antigens has been performed as follows: The O inagglutinability of the living strain under test was first examined on a slide with undiluted O serum. If the strain was completely O inagglutinable, it was agglutinated in OK immune sera corresponding to the K antigens of the O group. If the strain did not possess any of the K antigens of the O group in question, the occurrence of K antigens of other O groups was then investigated, with the exception of the A antigens which were examined in the O groups 8 and 9 only.

If the strain was not O inagglutinable, it was examined in OK immune sera in test tubes using both the living and heated strain. If the OK immune serum had been prepared with a strain belonging to the same O group as the strain under test, the latter was examined using the pure K serum. Unabsorbed sera were employed when the tested strain belonged to a different O group or when owing to the lack of suitable strain pure K serum was not obtainable.

All the results obtained by slide agglutination were confirmed by tube tests.

RESULTS

Of the 483 strains examined, 474 gave the reactions typical of E. coli (Mt $\frac{+}{+}$, Indole +, H_2S —, Gel. —, Cit. —, V-P. —, MR +, urea —), while 9 of the strains deviated in one or two of the reactions. It was found possible to identify 153 (31.7%) of the strains serologically. One (11: L4: 4) of these strains gave a positive Voges-Proskauer reaction.

One hundred and twenty-eight (31.0%) of the 408 strains isolated from urine samples could be serologically identified, and 25 (33%) of the 75 strains isolated from the pus samples. One hundred and fifty (30.6%) of the strains belonged to the first 25 O groups; one further strain was identified as E.coli 55:—:7, one as 86 : K?: — (not B7) and one as 126 : B16. The identity of this strain was kindly confirmed by Dr. Joan Taylor of the Central Public Health Laboratory, Colindale, London.

TABLE 1

THE ANTIGENIC FORMULAE AND THE HAEMOLYTIC POWER OF THE DIAGNOSED STRAINS

A Strains conforming with the diagnostic schema
B Strains deviating from the diagnostic schema in respect of K and H antigens

Group		Antigens			Number of Strains		Number of Haemolytic Strains
		O	K	H			
1	A	1	1 L	7	15		2
		1	1 L	—	1		3
	B	1	?	—	1	17	
2	A	2	1 L	7	4		
		2	1 L	—	3		
		2	2a, 2b L	1	4		4
		2	5 L	4	5		3
	B	2	1 L	1	1		
		2	5 L	7	1		
		2	5 L	—	3		2
		2	13 L	1	5		5
		2	13 L	6	2		1
		2	23 L	1	1		1
		2	45	1	1		1
		2	45	4	1		1
		2	?	1	5		5
		2	?	4	3		3
		2	?	7	2		1
		2	—	6	1	42	1
3	B	3	13 L	?	1		
		3	13 L	—	1		
		3	?	?	1	3	
4	A	4	6 L	5	1		1
		4	12 L	5	15		15
	B	4	12 L	—	4		4
		4	?	4	1		
		4	?	5	3		3
		4	?	—	1		
		4	—	5	4		3
		4	—	16	1		1
		4	—	—	1	31	1
5	B	5	7 L	10	1		
		5	?	—	1	2	

Group	Antigens			Number of Strains		Number of Haemolytic Strains
	O	K	H			
6 A	6	2a, 2c L	1	1		1
	6	13 L	1	3		2
B	6	5 L	1	1		
	6	15 L	1	1		1
	6	53	1	1		1
	6	?	—	1		1
	6	?	1	3	11	1
7 A	7	1 L	—	3	3	
8 B	8	20 L	?	1		
	8	45	?	1		
	8	?	9	1	3	
10 B	10	5 L	—	2		
	10	*	?	1	3	
11 A	11	10 L	10	1		
B	11	4 L	4	3		
	11	52	4	1		
	11	?	—	1	6	
12 B	12	?	—	2	2	
13 A	13	—	11	3	3	
15 B	15	20 L	—	5		
	15	24 L	—	1		
	15	?	—	1		
	15	—	?	1	8	
16 B	16	?	—	1	1	
18 B	18	5 L	1	2		2
	18	5 L	—	1		
	18	6 L	?	1		1
	18	?	—	1	5	1
19 B	19	?	—	1	1	
20 B	20	*	—	1	1	
21 A	21	14 L	4	1		
B	21	24 L	31	1		1
	21	?	4	1		
	21	—	?	1		
	21	*	?	1	5	1
23 B	23	14 L	6	1	1	
24 B	24	?	—	1	1	
25 B	25	?	—	1	1	

* K antigen determination failed.

TABLE 2

CLASSIFICATION OF THE STRAINS ACCORDING TO THEIR CONFORMITY WITH THE DIAGNOSTIC ANTIGENIC SCHEMA

O Group	Serologically Identified Strains	Number of Strains
1—25	A. Strains conforming with the diagnostic schema	60
	B. Strains deviating from the schema[1]	
	1. In respect of K	46
	2. In respect of H	13
	3. In respect of both K and H	31
55	Strains identified,	1
86	but not included	1
126	in the diagnostic schema	1 153 (31.7%)
	Unidentified strains	330
	Total	483

[1] 3 unsuccessful K antigen determinations included.

The number of strains conforming with the antigenic schema was 60. The remaining 90 strains, including 3 strains whose K antigen determination failed, deviated in respect of the K and H antigens (Tables 1 and 2).

Of the strains belonging to the first 25 O groups, 134 (89.3%) possessed a K antigen. K antigens belonging to the O group in question were found in 73 strains, other known K antigens were possessed by 30 strains, while for 32 strains the K antigens (K?) could not be determined. Twelve strains possessed no K antigen and the determination failed for three strains (Tables 1 and 2).

One hundred and ten (73.4%) strains were found to be motile. The H antigens of 92 strains were those corresponding to the O group in question, other known H antigens were found in 10 strains and the determination of H antigens of the remaining nine strains did not succeed with the H immune sera 1—33.

The most frequently occurring O-groups were 1, 2, 4 and 6. Sixty-six per cent of the groupable strains belonged to these groups.

With one exception (126 : B16) strains belonging to E.coli types associated with infantile diarrhoea were not found in the present material.

Of the 153 serologically identified strains, 75 (49.1%) were haemolytic. One hundred and eighty-one of the unidentified strains also produced haemolysis (56.3%).

COMMENT

The elaboration of the diagnostic antigenic schema of Kauffmann-Knipschildt-Vahlne is a great advance in the serology of Escherichia group. By this means it is possible to classify a rather high percentage of Escherichia strains.

The proportion of strains from the urine and pus samples that were identified are practically equal, 31.0 and 33.3 per cent. For comparison it may be mentioned that Leppänen (13) was able to identify 34 per cent of the 154 strains he isolated from normal faeces samples and that Levanto (14) was able to type 33 per cent of the 82 E. coli strains he isolated from gastric juice.

Haemolytic strains were more frequently found in the O groups 2, 4 and 6, but almost equal numbers of haemolytic strains were found among the identified and unidentified strains.

SUMMARY

By using immune sera prescribed in the diagnostic antigenic schema of Kauffmann-Knipschildt-Vahlne, immune serum O 78 and sera for Escherichia strains 111 : B4, 55 : B5, 26 : B6, 44 : K?, 86 : B7, 126 : B16 and Canioni and immune sera H 1—33, it has been found possible to type 153 strains (31.7%) out of a total of 483 Escherichia strains isolated from urinary infections and suppurative conditions.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY,
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TOXOPLASMOSIS IN NORWAY RATS IN HELSINKI

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Toxoplasmosis is generally considered a zoonosis. It is still obscure, however, which of the animals are the most common source of the human Toxoplasma infection, though it would seem natural to try to find the source among those living in close contact with human beings. Various investigations have been performed to estimate the frequency of infection in domestic animals, such as dogs and cats.

As early as 1914, Sangiorgi (8) found Toxoplasma in an albino rat that died spontaneously. Perrin, Brigham and Pickens (4) were the first to study wild rats systematically with regard to Toxoplasma. Their parasitologic and histologic investigations revealed that 8.7 per cent of 160 rats from Savannah, Georgia, were Toxoplasma carriers. Laven and Westphal (3) carried out serologic tests on 34 rats from Hamburg and reported that five of them had a dye test titer of 1/25 or over. Eyles (1) isolated six Toxoplasma strains from 100 rats in Memphis, Tennessee. He also performed serologic investigations on the same series with the following results:

Serum Dilution	Dye Test Positive	Dye Test Negative
1/1	12	80
1/16	6	
1/256	1	
1/4096	1	

In spite of negative reactions to the dye test, Toxoplasma was isolated from two rats. Eyles (2) conducted experiments to determine whether Toxoplasma infection in white rats is really reflected by a positive dye test. All the rats showed positive reactions, and the median peak titer was 1/4000. After a year the median titer was 1/16.

Present Investigation. — The present series comprised 108 rats of the species *Rattus norvegicus*, captured alive in Helsinki. The dye test and complement-fixation test were performed on all of them. In a part of the blood samples, the occurrence of *Trypanosoma lewisi* was studied microscopically and the presence of Leptospira antibodies was determined by means of an agglutination-lysis test against *Leptospira icterohaemorrhagiae* AB to find any possible correlation between these infections and Toxoplasma antibodies. The results are shown in Table 1.

TABLE 1

RESULTS OF TESTS ON RATS

Method of Study	No. of Rats	Positive Sera	
		%	Distribution of Titer
Dye test	108	12	11 (1/4), 1 (1/16), 1 (1/64)
Toxoplasma complement-fix.	108	4	2 (1/4), 2 (1/8)
Trypanosoma in blood ..	68	38	
Leptospira antibodies ..	97	52	$\geq$1 : 50

The dye test was performed according to the method described by Sabin *et al.* (7). The sera were inactivated for 30 min. at 56° C before the test. Twelve per cent of the samples were positive in a dilution of $\geq 1/4$. Tests with undiluted sera were not performed to save the serum for other investigations. The majority of positive samples was positive only in the first dilution of 1/4, other samples were positive in titers 1/16 and 1/64.

The Toxoplasma complement-fixation test was conducted with antigen prepared in chick embryos according to Warren and Russ (10) and Sabin (6). Fourteen sera gave anticomplementary reactions, and one serum proved to be nonspecific for the chick proteins in antigen, so that 15 sera could not be studied. The complement-fixation test gave positive results in four samples, the titers being 1/4—1/8. Only one of these sera was dye test positive (titer 1/4).

Westphal assumed (11) that Toxoplasma and Trypanosoma are close relatives and reported that the Toxoplasma complement-fixation test is also positive in Trypanosoma infections. Table 2 shows that there is no difference in the positiveness of the dye test and the Toxoplasma complement-fixation test for Trypanosoma positive or negative groups in the blood. Since Trypanosoma antibodies were not studied in our series, we cannot say whether a correlation exists between them and the Toxoplasma complement antibodies.

TABLE 2

CORRELATION OF TOXOPLASMA ANTIBODIES TO TRYPANOSOMA INFECTION AND LEPTOSPIRA ANTIBODIES

Method of Study	No. of Sera	Dye Test Positive		Compl. Positive		Anti-compl.	
		No.	%	No.	%	No.	%
Trypanosoma +	26	3	12	2	8	7	27
Trypanosoma —	46	6	14	2	5	7	17
Leptospira antibodies +	51	3	6	2	4	7	14
Leptospira antibodies —	46	7	15	1	2	6	13

TABLE 3

FREQUENCY OF INFECTION FOR TWO PLACES OF CAPTURE

Places of Capture	No. of Sera	Dye Test Positive		Trypanosoma in Blood		Leptospira Antibodies	
		No.	%	No.	%	No.	%
Place A Zool. Gardens	48	4/48	8	21/47	45	34/48	71
Place B Stable of Institute of Vet. Medicine	28	3/28	11	2/14	14	7/28	25

Table 3 shows the infection frequency for two different places of capture. In Place A, the rat strains are abundant and there are no hindrances to their breeding. In Place B, rats are destroyed by means of poisons and traps at certain intervals and their number is small. The frequency of infection for both Trypanosoma and Leptospira is remarkably greater in Place A than in Place B. On the other hand, a similar difference cannot be noted in the occurrence of Toxoplasma antibodies, so that the abundance of rat strains does not seem to be a promoting factor in toxoplasmosis. Laven and Westphal (3) were of the opinion that Toxoplasma may be carried by ectoparasites. We did not study ectoparasites in rats, but it

seems unlikely, that *Nosopsyllus fasciatus*, the vector of *Trypanosoma lewisi*, should carry Toxoplasma (*Cf.* Table 2.).

Discussion. — The low values for dye-test titers are most conspicuous in the present study. Since the low titers may be nonspecific, it seems obvious that toxoplasmosis is a rather rare disease among rats in Helsinki. It can be thought that the rats infected with Toxoplasma are so ill that they do not move and are not caught with traps. The results of experiments on albino rats, which have proved to be rather resistant to Toxoplasma infections (2, 5, 9), do not, however, favor this assumption. It is also possible that the antibody production of wild rats is poor and that they would, in this respect, differ from the albino rats studied serologically by Eyles (2).

Summary. — The Toxoplasma antibodies were studied in the sera of 108 rats (*Rattus norvegicus*) captured in Helsinki. The dye test was positive in 12 per cent in a dilution of $\geq 1/4$. The titers were low, the highest being 1/64. The complement-fixation test gave positive results in 4 per cent.

No correlation was found between Toxoplasma antibodies and the occurrence of *Trypanosoma lewisi* in the blood (38 per cent) and the presence of antibodies against *Leptospira icterohaemorrhagiae* (52 per cent).

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FROM THE STATE LABORATORY OF SEROLOGY AND BACTERIOLOGY, KUOPIO

THE RESISTANCE OF STAPHYLOCOCCI TO ANTIBIOTICS AND SULFA DRUGS

by

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During recent years, increasing attention has been paid to the clinical significance of staphylococcal infections (2, 7, 8, 12). Many clinically disappointing results with penicillin and other antibiotics have been due to the appearance of resistant staphylococcal strains. Though it is generally known that clinical results do not always parallel the laboratory tests for sensitivity (8), the determination of bacterial sensitivity is an important factor in the effective use of chemotherapeutic and antibiotic preparations (6). The present study was undertaken for the purpose of evaluating the *Staphylococcus* problem with regard to the sensitivity to various antibiotics and sulfa drugs of strains isolated from Sept., 1951, to Dec. 1954.

MATERIAL AND METHODS

The strains of staphylococci tested in this study were obtained, usually in pure culture, from patients at various hospitals in Middle and Eastern Finland. A minority of the strains (46 or 16.4 per cent) was isolated from ambulatory out-patients. Each of the strains tested for sensitivity was derived from a single colony of staphylococci. The sources of the isolated strains are listed in Table 1.

The method for determining the sensitivity of the isolated organisms to the various antibiotics was similar to the disc plate method

TABLE 1

SOURCES OF STRAINS TESTED

Sources	No. of Strains
Abscesses and infected cavities	130
Discharge from the ear	45
Urine	39
Skin (vesicles, wounds, exudative lesions)	27
Respiratory tract (nose, throat, sputum)	26
Blood	9
Discharge from the eye	2
Osteomyelitis	2
Total	280

described by Kaipainen (6). Concentrations of the antibiotics used were as follows:

Penicillin	20 units/ml
Dihydrostreptomycin	500 μg/ml
Chlortetracycline	500 μg/ml
Oxytetracycline	500 μg/ml
Chloramphenicol	500 μg/ml
Erythromycin	500 μg/ml

Sulfathiazole was employed until 1953 and sulfisoxazole (Gantrisin) thereafter. The concentration of the sulfa drugs was 2 mg/ml.

Staphylococci were considered sensitive to the preparation tested when the zone of inhibition was $\geq$ 30 mm, less sensitive when the zone was 15 to 25 mm and resistant when the zone was $\leq$ 10 mm.

RESULTS

The relative incidence of staphylococci did not increase between 1951 and 1954, as can be seen from Table 2.

TABLE 2

DETERMINATIONS OF BACTERIAL RESISTANCE, 1951—1954

Year	Total No. of Tests on Bacterial Sensitivity	Tests on Sensitivity of Staphylococci	
		No.	% of Total Tests
1951—52	112	45	40.1
1953	198	91	45.5
1954	342	144	42.1
Total	652	280	42.9

Staphylococcal sensitivity to the various antibiotics and sulfa drugs is shown in Table 3.

TABLE 3

STAPHYLOCOCCAL SENSITIVITY TO ANTIBIOTICS AND SULFA DRUGS

Antibiotics and Sulfa Drugs	Grade of Resistance	No. of Strains	% of Strains
Penicillin	S	62	22.1
»	LS	34	12.1
»	R	184	65.8
Dihydrostreptomycin	S	77	27.5
»	LS	71	25.4
»	R	132	47.1
Chlortetracycline	S	63	22.5
»	LS	78	27.9
»	R	139	49.6
Oxytetracycline	S	66	39.0
»	LS	61	36.0
»	R	42	25.0
Chloramphenicol	S	111	39.6
»	LS	100	35.7
»	R	69	24.7
Erythromycin	S	45	38.1
»	LS	54	45.8
»	R	19	16.1
Sulfa drugs	S	9	3.5
»	LS	11	4.2
»	R	241	92.3

S — sensitive
LS — less sensitive
R — resistant

Variations in staphylococcal sensitivity to the antibiotics and sulfa drugs used in the study may be seen in Table 4.

DISCUSSION

The results shown in Table 3 must be taken with certain reservations, since in the disc plate method the size of the inhibition zone does not depend only on staphylococcal sensitivity but on other factors as well (8). This method seems to give an unfavorable picture of the effectiveness of aureomycin, in particular (6).

TABLE 4

VARIATIONS IN STAPHYLOCOCCAL SENSITIVITY TO ANTIBIOTICS AND SULFA DRUGS, 1951—1954

Years	Antibiotics and Sulfa Drugs	No. of Strains	Sensitive	Less Sensitive	Resistant	% of Resistant Strains
1951—52	Penicillin	45	20	1	24	
	Dihydrostreptomycin	45	10	9	26	
	Chlortetracycline	45	4	5	36	1951—53
	Oxytetracycline	45	14	22	9	
	Chloramphenicol	45	29	7	9	
1953						
	Penicillin	91	22	12	57	60
	Dihydrostreptomycin	91	34	28	29	40
	Chlortetracycline	91	17	24	50	63
	Oxytetracycline	91	32	28	31	29
	Chloramphenicol	91	44	30	17	19
	Sulfa drugs	72	2	1	69	96
1954						1954
	Penicillin	144	20	21	103	72
	Dihydrostreptomycin	144	33	34	77	53
	Chlortetracycline	144	42	49	53	37
	Oxytetracycline	33	20	11	2	
	Chloramphenicol	144	38	63	43	30
	Erythromycin ..	118	45	54	19	16
	Sulfa drugs	144	7	10	127	88

On the other hand, the results listed in Table 4 are comparable *inter se*, as the method was the same throughout the period of study.

The staphylococci tested were rather sensitive to erythromycin. This was not unexpected, as the drug has proved to be highly effective against all staphylococci, irrespective of their sensitivity or resistance to other antibiotics (1, 2, 3, 4, 5, 14). Erythromycin is still little used in Finland, and staphylococci have not as yet become resistant to it, although such resistance is a common phenomenon elsewhere (1, 4, 5, 11). The effectiveness of erythromycin becomes even more evident in view of the fact that 20 of the 25 staphylococcal strains resistant to all but one of the antibiotics were sensitive to erythromycin. Four of the 25 strains were

sensitive to chloramphenicol, and one was sensitive to penicillin. However, strains resistant to erythromycin were also encountered. Their number (16.1. per cent) is in agreement with that reported by Spink (12): 28 (18.3 per cent) of the 153 strains tested by him were resistant.

The fact that stpahylococci in the present study were rather sensitive to chloramphenicol also agrees with the results reported by Spink (12). He found chloramphenicol to be the most effective of the antibiotics and pointed out that chloramphenicol-resistant strains were encountered infrequently. On the basis on their tests with 500 staphylococcal strains, Finland and Haight (2) listed the following antibiotics in the order of their effectiveness: erythromycin, aureomycin, oxytetracycline, bacitracin, streptomycin and, not until the sixth, chloramphenicol.

Spink (12) also stressed the fact that, while the number of staphylococcal strains resistant to chlortetracycline and oxytetracycline increased year after year, the number of strains resistant to chloramphenicol decreased considerably. According to Finland and Haight (2), a cross-resistance occurs in vitro between chlortetracycline and oxytetracycline on the one side and chloramphenicol on the other, though it is not usually so striking.

In the present study, chloramphenicol was more or less effective against 86 of 139 chlortetracycline-resistant strains. Chlortetracycline, on the other hand, was effective against only 10 of 68 chloramphenicol-resistant strains. Of the 42 oxytetracycline-resistant strains, chloramphenicol was effective against 27. Oxytetracycline, for its part, was effective against only six of 69 chloramphenicol-resistant strains.

The effectiveness of chloramphenicol on our staphylococci is perhaps due to the fact that this antibiotic was not generally used in Finland for the treatment of various infections (including staphylococcal infections) until recently. This assumption is further supported by the observation that the number of chloramphenicol-resistant strains did not show any distinct increase prior to 1954.

It became evident in the present study that staphylococci were more sensitive to oxytetracycline than to chlortetracycline. The latter proved to be a highly ineffective antibiotic in my series. This is in disagreement with the results of Finland and Haight (2), who reported that a cross-resistance probably plays a considerable

role in the case of aureomycin and oxytetracycline. The parallel effectiveness of these drugs cannot be observed clearly in the study by Spink (12).

The increasing sensitivity of staphylococci to chlortetracycline was conspicuous during the test period. Between 1951 and 1953, 63 per cent of the 136 strains tested were chlortetracycline-resistant, while only 37 per cent of the 144 strains tested in 1954 were resistant to this antibiotic. Spink (12) reported that 62.7 per cent of the 153 staphylococcal strains tested in 1953 were chlortetracycline-resistant.

The problem of the changing reactions of staphylococci is difficult to solve. It may be assumed that the increased use of other antibiotics (erythromycin, oxytetracycline, chloramphenicol) caused a corresponding decrease in the use of chlortetracycline and that staphylococci, being thus released from its effectiveness, became gradually sensitive to it. In other words, the earlier resistance of staphylococci may have been an occasional and reversible phenomenon (12, 13).

It is obvious that the sensitivity of staphylococci to dihydrostreptomycin is diminishing also in Finland. Forty per cent of the 136 strains tested in 1951—53 were dihydrostreptomycin-resistant, the corresponding figure for the 144 strains tested in 1954 being 53 per cent. According to Spink (12), the occurrence of streptomycin-resistant strains was 48 per cent in 1951 and 65.3 per cent in 1953.

The increased resistance of staphylococci to penicillin is generally known, and numerous reports have been published on this subject. Spink (12) noted that all staphylococcal strains tested in 1940 were highly sensitive to penicillin, while 50 per cent of the strains tested in 1951 and 62.7 per cent of those tested in 1953 were penicillin-resistant. Finland and Haight (2) found that 124 (24.8 per cent) of the 500 staphylococcal strains tested by them were sensitive to penicillin. Eleven strains (2.2 per cent) were less sensitive, 365 (73 per cent) being resistant. An increase in the number of staphylococcal strains resistant to penicillin was observed in the present study; 60 per cent of the 136 strains studied between 1951 and 1953 and 72 per cent of the 144 strains studied in 1954 were penicillin-resistant.

Staphylococci proved to be highly resistant to the sulfa drugs. Clear differences could not be seen in their sensitivity to different sulfa drugs through the period of study.

»Reports do not show that staphylococcal strains resistant to all the available antibiotics occur frequently» (8). In the present series, only 17 (6 per cent) of the 280 staphylococcal strains tested were resistant to all the antibiotics used. The number is rather small perhaps because the tests were performed with many antibiotics. If we exclude the erythromycin experiments performed in 1954, we find that the number of staphylococcal strains resistant to other antibiotics used in the study tends to increase notably. Ten (7 per cent) of the 136 strains tested in 1951—53 and 27 (19 per cent) of the 144 strains tested in 1954 were resistant to all the preparations involved.

It is believed that penicillin-sensitive strains of staphylococci generally tend to be associated with lesions which are closed or deep-seated, regardless of whether the process is an acute or chronic one, while the resistant strains are usually associated with superficial infections (10). However, various investigators have not agreed with this concept (8, 9). Nor were notable differences in the sensitivity of staphylococcal strains isolated from various sources found in the present study.

SUMMARY

Two hundred and eighty strains of staphylococci isolated from patients with acute or chronic infections during the years 1951—54 were tested for their sensitivity to antibiotics and sulfa drugs by the disc plate method.

The most striking finding was a marked increase in the incidence of penicillin- and dihydrostreptomycin-resistant strains during the period of study. About three-fourths of the strains isolated in 1954 were resistant to penicillin and a half of the strains were resistant to dihydrostreptomycin.

Erythromycin-resistant strains were rather rare (16 per cent). About one-fourth of the strains were resistant to chloramphenicol and to oxytetracycline, but a significant increase occurred in the incidence of strains resistant to chloramphenicol. On the other hand, the sensitivity of staphylococci to chlortetracycline showed a tendency to increase, though reasons for this still remain obscure.

There was no correlation between resistance to chloramphenicol and resistance to chlortetracycline and oxytetracycline.

The source of the strains did not influence their resistance to penicillin.

A majority (92 per cent) of the staphylococcal strains was resistant to sulfa drugs. No difference was noted in their effectiveness.

The rapid changes in the sensitivity of staphylococci to antibiotics necessitate frequent testing of the sensitivity of staphylococci.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI, AND THE MUNICIPAL BACTERIOLOGICAL LABORATORY, HELSINKI

SEPARATION OF DIFFERENT PHASES OF DIPHASIC SALMONELLAE

A RAPID AND SIMPLE METHOD

by

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Diphasic Salmonella cultures often occur in only one phase when recently isolated. If only the non-specific phase is present, it is necessary to obtain a specific phase to identify the culture. The Gard plate method or its many modifications (2.3) with swarm agar are generally used. However, with the modifications the specific phase is established after two days at the earliest, and with the Gard plate method the need of antiserum is relatively large.

The purpose of the study here reported was to develop a rapid and simple method giving adequate results with a minimum serum demand, by adapting the Braun and Howell method for separation of smooth bacterial types from mixed populations in a sintered U-tube (1).

During the epidemic caused by S. typhi murium, 13 strains were selected, which in pure culture gave slide agglutination with the non-specific serum 1.2.3.5. only, but not with the specific serum i. All strains were also tested with Gard plates.

A U-tube with an inner diameter of 5 mm, in which a glass sinter with a mean porosity of 15 microns separated both sides, was sterilized in a dry-air sterilator, and 3 ml of plain nutrient broth was pipetted to both sides of sinter. 0.06 ml of antiserum 1.2.3.5.

(with a titre of 1: 6400) was pipetted to one side and a loop-full of the test strain from an agar culture was inoculated to the same side. After 4 hour's incubation at 37° C a loop-full from the other side was inoculated to an agar plate and was incubated overnight. Slide agglutination from the colonies was then performed with both sera. The results are given in table 1.

TABLE 1

RESULTS WITH SLIDE AGGLUTINATION BEFORE AND AFTER SEPARATIONS

Strain	Agglutination before		Incubation Time in U-tube Hours	Agglutination after	
	1.2	i		1.2	i
1	++	—	24	—	++
2	++	—	4	±	++
3	++	—	4	—	++
4	++	—	4	—	++
5	++	—	4	—	++
6	++	—	5	—	++
7	++	—	5	+	++
8	++	—	5	—	++
9	++	—	5	+	++
10	++	—	5	—	++
11	++	±	5	—	++
12	++	—	5	±	++
13	+	—	5	—	++

DISCUSSION

With the method used, the non-specific antiserum added to the broth agglutinated the bacteria which were in a non-specific phase and prevented them from entering through the sinter to the other side, while the bacteria which were in a specific phase grew freely. Separation was possible with all the strains, and although the non-specific antiserum also gave slight agglutination after manipulation in four cases, agglutination with the specific antiserum was prevalent. The method seems to be useful for Salmonella identification, as the results are readable on the second day and as the need of antiserum is relatively smaller than in Gard plates. It might be still reduced by using smaller tubes or V-shaped tubes with the sinter imbedded at the angle and smaller amount of broth.

SUMMARY

A simple rapid method is described in which with a sintered U-tube the separation of different phases of diphasic Salmonellae is possible with a minute antiserum requirement.

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FROM THE STATE SERUM INSTITUTE, HELSINKI

A NEW SEROLOGICAL TEST FOR TUBERCULOSIS

by

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We present a new serological test for tuberculosis. The technique of the test is based on the technique of the VDRL tube test, which is well known in syphilis serology.

Antigen. — An at least six weeks old tubercle bacillus culture on Sauton medium is filtered and the bacillus mass washed once with distilled water. This moist bacillus mass is extracted with absolute ethanol. The time of extraction is seven days, the temperature is the room temperature, and the ratio of the bacillus mass to the absolute ethanol is one gram to 20 ml. The mixture is filtered through a Seitz filter. The filtrate contains c. 2 mg per ml of the tubercle bacillus extract in absolute ethanol. The antigen is prepared by adding one part of this filtrate to three parts of absolute ethanol containing 0.4 per cent of egg lecithin and 1.2 per cent of cholesterol.

Technique and Performance of Test. — The preparation of the final antigen emulsion and the performance of the test follows the VDRL tube test technique as described in the Manual of Serological Tests for Syphilis 1949, with the following exceptions. The centrifugation must be performed at a speed of at least 2200r. p.m. and a radius of 25 cm. Shaking after centrifugation must be done with the finger tip or for three minutes in a Kahn shaking machine with a 3.5 cm stroke and 200 oscillations per minute.

All the sera must be inactivated for 30 minutes in a water bath at 56 C before testing.

The positive reaction is similar to the positive reaction in the VDRL tube test. In a negative tube the typical »silken swirl» remains unchanged.

We have examined hitherto:

10 sera from patients with pulmonary tuberculosis in Nummela Sanatorium, 8 of which were positive and 2 negative;
144 sera chiefly from patients with pulmonary tuberculosis in Helsinki Sanatorium, 86 of which were positive (60 per cent), 15 weakly positive (10 per cent) and 43 negative (30 per cent). Among the negative cases there was one case of Boeck's sarcoid and one case of suspected bone tuberculosis;
36 sera from blood donors, one of which weakly positive;
15 sera from patients in a medical hospital, one of which weakly positive;
16 sera from pregnant women, all negative;
13 sera from scarlet fever cases, all negative;
4 sera from pneumonia cases, one of them positive (1 dil);
6 sera from pulmonary carcinoma cases, one weakly positive;
10 sera which were sitolipin (and cardiolipin) positive and which were all positive.

All the sera were titrated and the highest positive dilution was 256, the ninth tube.

To summarise, there were 154 sera of TB material, 94 of which were positive, 15 weakly positive and 45 negative; 90 sera from control cases, one of which was positive and 4 weakly positive; 10 sera which were sitolipin positive (syphilitic?) and which all were positive in the present test.

Estimation of the value of the presented test for clinical use is under study, as well as the purification of the antigen and development of the technique.

An observation which may be of some topical interest is the big difference in the results obtained with active and inactivated sera. The active serum very often gives a positive result even for a healthy person. Table 1 shows some results obtained in titrations of the same serum when used active and inactivated.

Table 1

Serum No.	Active Serum					Inactiv. Serum				
	1dil	2d	1d	8d	16d	1dil	2d	4d	8d	16d
1	+	+	+	wp	—	+	+	+	wp	—
2	+	+	+	+	—	—	—	—	—	—
3	—	—	—	—	—	+	+	+	+	—
4	—	—	—	—	—	—	—	—	—	—
5	—	wp	+	+	+	+	+	+	+	+
6	—	wp	+	—	—	+	+	+	+	—
7	+	+	+	wp	—	wp	—	—	—	—
8	+	+	wp	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	—	—	—

Sera Nos. 1—7 are from TB cases and sera 8 and 9 from healthy persons. Some sera which were positive when active were negative after inactivation, and vice versa. In addition there was a very marked inhibition zone in some active sera.

FROM THE STATE SERUM INSTITUTE, HELSINKI

THE VARIATION IN THE RESULTS OF HEMAGGLUTINATION AND HEMAGGLUTINATION INHIBITION TESTS WITH INFLUENZA AND MUMPS VIRUSES CAUSED BY THE RED CELLS OF DIFFERENT FOWLS

by

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Investigations of the factors affecting the hemagglutination induced by influenza viruses soon established that some variation in the results was due to the different chicken cells used (9). The differences in the titres obtained, however, were quite small (10, 13). But in hemagglutination inhibition titrations the differences were observed to be greater. The differences in the titres of immune sera with different cells could be 2—12-fold (2, 4, 10, 18). In the titrations of unspecific inhibitors of the sera the differences could be greater still (1, 2). In titrating the different influenza virus strains the titre differences between the different cells seemed to vary (2). The cells which in virus titrations gave the highest titres seemed to yield the lowest inhibition values (2). The greatest differences between the different cells were obtained in titrations of the so-called Francis inhibitor (8). Here the difference in titre could be even 60-fold (1). In titrating the inhibitor prepared on meconium, too, some degree of variation between fowls was found (7).

In the investigation of hemagglutination phenomena induced by the mumps virus the variations between the cells of the different chickens attracted less attention. With chick red cells hemagglutination titres were found to vary approximately fourfold (3). However, the differences were not correlated with the smaller

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differences observed with influenza virus strains, i.e. the cells giving low titres did not give low titres with the mumps virus as well, nor did those giving high titres produce the same result with the mumps virus (3). It was also found in our laboratory that the cells of different chicks in certain experimental conditions gave widely divergent results in mumps virus titrations (15). This is in conformity with earlier observation (12).

The following investigation shows the range of variation chick red cells may induce in certain hemagglutination tests. Special attention has been devoted to the titration of meconium inhibitor with two influenza virus strains. The significance of different chicken cells for hemagglutination tests with mumps virus has also been studied.

VIRUS STRAINS AND METHODS EMPLOYED

Virus Strains. 1. Influenza A/Finland/1/51 E 33—37 (Scandinavian), 2. Influenza B Lee F 8 M 139 E 131—179. 3. Mumps virus, Enders strain E 31—40 (in our laboratory).

Indicator Viruses. Indicators were prepared from the influenza strains employed by heating virus allantoic fluids for 30 minutes at + 56° C.

Preparation of Virus Allantoic Fluids. — The technique used in infecting fertilized eggs and the treatment of allantoic fluids is that described previously (14).

Inhibitors. — Egg white inhibitor was prepared as described before (16). Meconium inhibitor was prepared according to Curtain, French and Pye (7). Lyophilised meconium inhibitor has been found to retain its titre for at least one year storage at + 4° C (21). The normal allantoic fluid employed as inhibitor was collected from eggs incubated for 12 days and used as fresh as possible after centrifuging for 10 min. at 1,500 r.p.m.

Red Cell Suspensions. — The suspensions were prepared by taking blood from chickens by cardiac puncture or from a wing vein into a syringe containing 3.2 per cent citrate, by washing the cells 3 times with sterile physiological saline solution and preparing a 10 per cent suspension of them after they had been centrifuged in a graduated centrifuge tube for 10 min. at 1,500 r.p.m. All the chicks involved were grown-up White Leghorns.

Hemagglutination Titrations. — Unless otherwise mentioned, plastic plates were used in the titrations (19). The dilutions were made in 0.25 ml of physiological saline solution buffered with phosphate (pH 7.4). The dilution coefficient was 2. 0.25 ml of 0.5 per cent red cell suspension was added to each dilution.

Hemagglutination Inhibition Titrations. — The inhibitor dilutions were made on plastic plates in a volume of 0.25 ml with 2 as the dilution coefficient. 4 agglutinating doses of virus in 0.25 ml were added to each dilution. After incubating for half-and-hour at room temperature, 0.5 ml of 0.5 per cent red cell suspension was added to each dilution; the results were read after 1 hour. Controls were used in every test to show that the amount of virus employed equalled 4 agglutinating doses and that the cells gave a negative result without the addition of virus.

Sonic Treatment. — The virus allantoic fluids were treated with sonic waves in the way described before (14).

Adsorption-elution Experiments. — Washed and centrifuged (at 1,500 r.p.m. for 10 min.) chick cells to be examined were added in sufficient quantity to give a 1.5—2.0 per cent suspension to virus-containing allantoic fluid. Agitating from time to time, the suspension was allowed to stand in refrigerator (+ 4° C), at room temperature (+ 18—21° C) or in a water bath (+ 37° C), depending on the test. The reagents for the different tests had been kept in advance at the corresponding temperatures. After 3, 10, 20, 30, 60 and 120 minutes, on agitation, an 0.5—1.0 ml sample was taken. The red cells were immediately centrifuged to the bottom at a corresponding temperature in all cases except after incubation at + 37° C at room temperature. The virus titre was determined from the supernatant with the cells of one and the same chicken.

RESULTS

Variations Shown by the Red Cells of Different Fowls in Hemagglutination Inhibition Titrations with Influenza Virus Strains. — Using the red cells of 31 chickens and 4 roosters a single meconium inhibitor preparation was titrated with Lee allantoic fluid inactivated for 30 min. at 56° C as the indicator. The results are given in Fig. 1.

Fig. 1 shows that the inhibition titres obtained with the cells

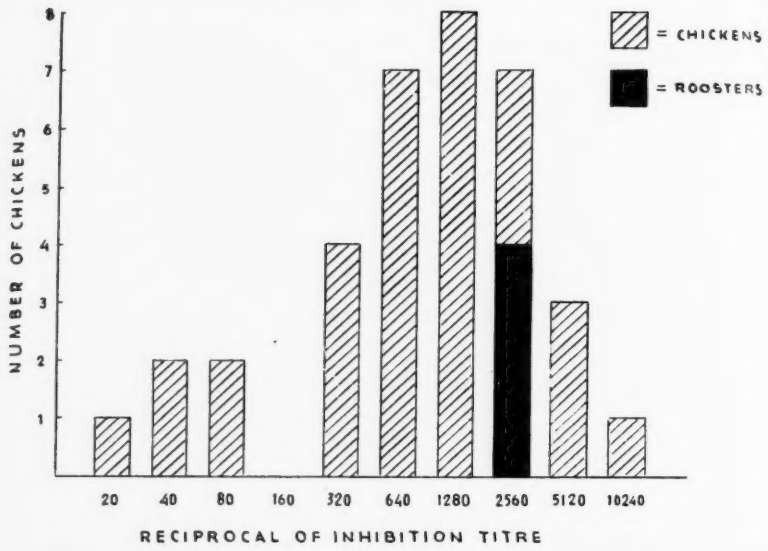

Fig. 1. — The titre of meconium inhibitor with the red cells of different chickens titrated with influenza B Lee inactivated for 30 min. at + 56° C.

of different fowls differ greatly, the maximal difference being over 500-fold. Differences of this order, however, are given by a small number only and the majority of the chickens gave considerably more homogenous results. The location of all the roosters at one point is probably coincidental. In addition, the figure shows that the greatest differences are towards the low inhibition titre. A small proportion, 5 out of 35 (14 per cent), thus seems to give very low titres when meconium is titrated with the Influenza B Lee strain as the indicator.

Six of the chickens examined were selected for further tests. 4 of them had given a low inhibition titre with meconium and 2 a high. Using their red cells, meconium was also titrated against strain A/Finland/1/51 (AF) as the indicator, and egg white inhibitor against the influenza virus strains mentioned as indicators. The results of these hemagglutination inhibition titrations are shown in Table 1.

Table 1 shows that the results obtained with different cells depend on both virus and inhibitor. Of the two viruses used as indicators the Lee-strain gives greater differences and of the two

TABLE 1

THE TITRES OF MECONIUM AND EGG WHITE INHIBITORS WHEN STRAINS INFLUENZA B LEE AND A/FINLAND/1/51 WERE USED AS INDICATORS

Chicken No.	Lee Meconium	Lee Egg white	AF Meconium	AF Egg white
21	20	1 280	1 280	8 000
22	40	1 280	640	8 000
16	40	1 280	1 280	8 000
23	80	2 560	640	8 000
103	2 560	2 560	1 280	8 000
20	10 240	5 120	5 120	8 000
Max.diff.	512-fold	4 fold	8-fold	0

Titres are expressed as reciprocals of the last dilution of inhibitor to show complete inhibition of hemagglutination.

inhibitors, meconium. Accordingly the greatest differences between cells are obtained when meconium is titrated against Lee. When egg white inhibitor was titrated against AF-strain no differences were observed. From Table 1, the cells e.g. of Chicken 20 could be called »inhibitor susceptible» (5, 17); this is revealed in tests other than the titration of egg white inhibitor with the AF strain as the indicator.

The following investigations are confined to the behaviour of the cells of Chickens 20 and 21, which gave the greatest differences, in certain other tests.

Varying the amount of indicator virus used in the test between 2, 4 and 8 agglutinating doses was not found to affect the difference yielded by the cells in meconium inhibitor titration.

Experiments with Mumps Virus. — As it had been observed previously that the red cells of different chickens could in certain conditions bring about considerable differences in the results of mumps virus titrations, the behaviour of the cells of Chickens 20 and 21 was studied in agglutination tests with the mumps virus.

In titrating Virus-containing Allantoic Fluids the »inhibitor susceptible» cells of Chicken 20 were found to give titres 2—8 times lower than the »inhibitor insusceptible» cells of Chicken 21.

After *sonic treatment of allantoic fluid* (14) the differences observable between the cells were smaller. The rise in titre of allantoic

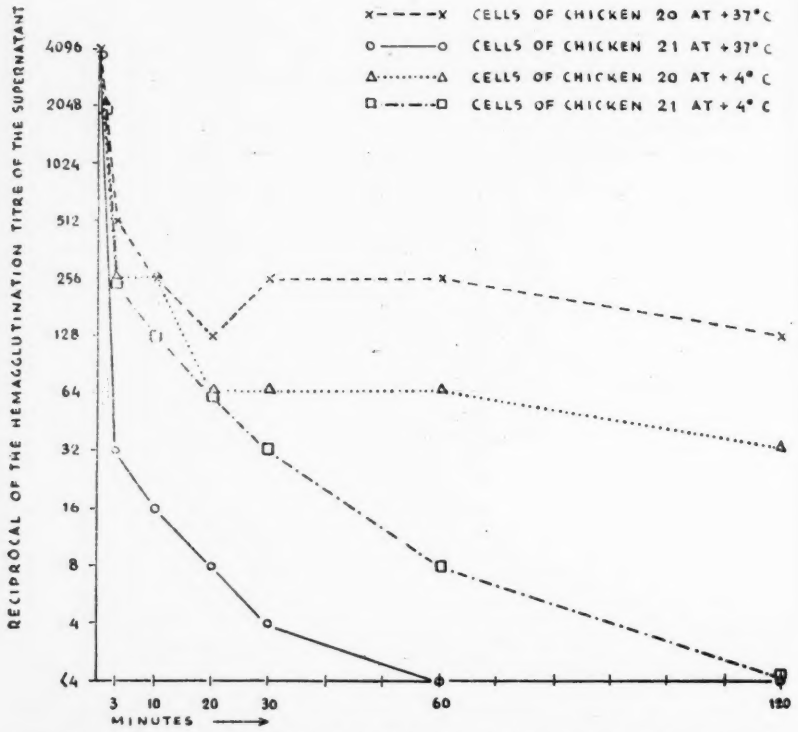

Fig. 2. — Adsorption experiments with inactivated influenza B Lee allantoic fluid. »Inhibitor susceptible» cells of chicken 20 and »inhibitor insusceptible» cells of chicken 21 have been added to the same allantoic fluid to give a 2 per cent suspension after which the residual hemagglutinins have been determined at the same time intervalls by using cells of a third chicken.

fluid containing mumps virus, following sonic treatment, seemed dependent on the cells used in the titrations. Using the »inhibitor susceptible» cells of Chicken 20, the titre of one allantoic fluid rose from 1/128 to 1/1024, with Chicken 21 cells from 1/512 to 1/1024. The experiments with other allantoic fluids gave corresponding results.

Using different temperatures in the agglutination tests in the presence of normal allantoic fluid (15) it was found that with the »inhibitor susceptible» cells of Chicken 20, no agglutination ($= 1/<40$) was observable at $+ 4°$ C or $+ 37°$ C, whereas the cells of Chicken 21 at the former temperature gave the titre $1/<40$ and at the latter 1/640. The test was carried out by adding 1 part of virus-con-

taining allantoic fluid to 9 parts of normal allantoic fluid; the mixture was kept for 30 min. at + 4° C, after which hemagglutination titrations of the same mixture were made at different temperatures, using glass tubes. The use of different temperatures and normal allantoic fluid in the agglutination test thus produced very considerable differences in the results given by the cells.

Adsorption-elution Tests. — When adsorption-elution tests were carried out with the cells of Chickens 20 and 21 it was found that the »inhibitor susceptible» cells of Chicken 20 did not measurably adsorb the mumps virus at + 4° C. In similar conditions and from the same allantoic fluid the »inhibitor insusceptible» cells of Chicken 21 adsorbed 75 per cent of the viruses. The maximum adsorption was at 30 minutes, after which slight elution could be observed. Adsorption-elution tests with the same technique but using active Influenza B Lee allantoic fluid revealed no differences between the cells of Chicken 20 and 21. Both cells adsorbed about 98 per cent of the viruses. When, however, inactivated Lee allantoic fluid was used the differences between the adsorption capacity of the cells were revealed. Fig. 2 shows the results in adsorption-elution tests with inactivated Lee allantoic fluid. In experiments in room temperature the differences between the cells were also clear.

Haematological Values of the Bloods of Different Chickens. — To ensure that it was not some blood disorder that was responsible for the different behaviour of the two types of cells, blood values used in clinical examinations were determined for the chickens involved: the erythrocyte count, haemoglobin content, colour index, haematocrit and erythrocyte diameter. There were practically no differences in the values obtained.

DISCUSSION

From the results reported, it seems obvious that the red cells of different chickens may produce considerable variations in the results of virus hemagglutination and hemagglutination inhibition tests (Fig. 1.). However, the picture is somewhat confused by the fact that the differences in the results with cells of different chickens are dependent on both virus strains and inhibitors. (Table 1.) When investigating more closely the cells of two chickens giving greatly divergent results it seemed as if the same cells were susceptible

to inhibition both in titrations of meconium and egg white inhibitors with influenza strains and in some tests with the mumps virus involving the allantoic fluid inhibitor. This may, however, be a coincidence as only cells of two chickens, one »inhibitor susceptible» and the other »inhibitor insusceptible», were examined in detail.

One factor to be considered in attempting an explanation of the phenomenon is the possible different affinity of the cells for virus (2, 20). This possibility is supported by the fact that the »inhibitor susceptible» cells failed to adsorb at + 4° C the mumps virus present in allantoic fluid and that they also had a weaker adsorption capacity than »inhibitor insusceptible» cells when inactivated Lee virus was in question.

Even if the affinity of cells of one chicken for virus is weak and that of the cells of the other chicken strong the differences between the cells remain usually small if no third factor competing for the virus, inhibitor, is present in the hemagglutination test. In addition, it can be considered that the affinity of the third factor, the inhibitor, for the virus plays some role. The diverging results with different cells in experiments with meconium inhibitor, in which inhibitor and inactivated virus were allowed to stay at room temperature for half an hour before adding the cells, are difficult to explain without assuming that the reaction between the indicator virus and inhibitor is reversible and that the binding forces between used indicator virus and meconium inhibitor are weak. If the inhibitor is »weak», as perhaps may be the case with meconium inhibitor, this causes a greater variation between different cells than if the inhibitor is so »strong» that the cells, whether »stronger» or »weaker» in adsorbing viruses, are incapable of competing with or detaching the virus from the »strong» inhibitor. The greatest heterogeneity in the results is probably encountered when both the inhibitor and the cells used have a weak affinity for the virus or the conditions of the test (e.g. temperature) weaken the affinity. As, in studying the effects of active viruses, their different ability to and rate of destroying inhibitors and cell receptors is also linked up with this competition of three factors in the hemagglutination inhibition reaction, the phenomenon confronting us becomes more complex still. (6, 9, 11, 15).

In some cases the dissimilar effect of the cells of different chickens can be reduced by destroying or otherwise inhibiting the ef-

fect of the inhibitor on the reaction. This can be done, e.g. in mumps virus studies, by the sonic treatment of the virus-containing allantoic fluid before hemagglutination titrations (14).

According to adsorption experiments the results given by the cells of different chickens can be influenced by changing the temperature of the test.

SUMMARY

The red cells of different chickens give greatly varying results in some virus hemagglutination and hemagglutination inhibition tests. This variation and its cause have been studied.

The cells of 35 different fowls gave over 500-fold differences in titres when meconium inhibitor was titrated with Influenza B Lee strain as indicator. The cells of 5 (14 per cent) of the fowls examined gave particularly low inhibition titres. With Influenza A/Finland/1/51 as the indicator the maximal difference in titres was 8-fold. Using egg white inhibitor the variation in the results due to the cells was slight.

»Inhibitor susceptible» and »inhibitor insusceptible» cells were also tested for the hemagglutination phenomena induced by the mumps virus; great differences were observed in the results obtained with them.

As a factor responsible for differences was considered, on the basis of adsorption tests, the different affinity of the cells for virus.

The significance of the results is discussed.

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FROM THE STATE SERUM INSTITUTE AND THE AURORA HOSPITAL OF THE CITY OF HELSINKI

ATOXIC DIPHTHERIA BACILLI AND THEIR SIGNIFICANCE IN CLINICAL DIPHTHERIA

by

ILARI RANTASALO

(Received for publication January 13, 1955)

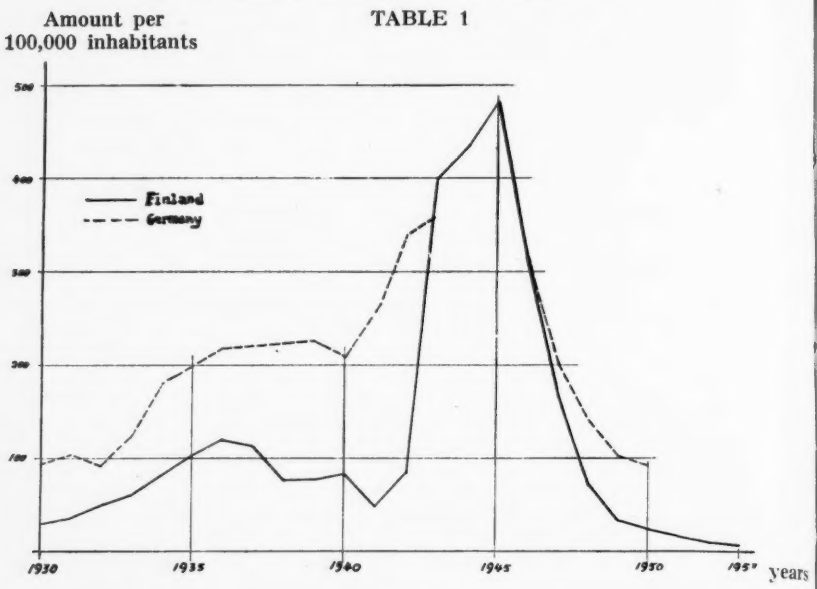
It is generally agreed that exotoxin produced by diphtheria bacilli causes toxic symptoms of diphtheria, such as nerve and heart muscle injuries, and that these injuries occur only in conjunction with exotoxin formation. Bergey's Manual makes the following remark on this matter: »A highly poisonous exotoxin is produced in fluid media. This toxin represents the principal disease-producing agency of the organism. Toxin production may fail in otherwise typical strains.» This toxin production is likewise lacking in so-called pseudodiphtheria bacilli, and, accordingly, the question arises where to draw the line between them and the diphtheria bacilli proper. Can we include among diphtheria bacilli in a more limited sense only those strains producing exotoxin? Another obscurity is the extent to which a strain's toxin production or complete lack of it are of epidemiological significance (6).

Earlier, the most usual method for the determination of toxin production was the guinea pig test. The plate method recently developed by Elek (2) and Ouchterlony (4) has been found to be noticeably quicker and cheaper for this purpose.

During World War II a large diphtheria epidemic lasting for several years attacked most European countries. Table 1 shows the annual number of cases in Finland and in Western Germany per 100,000 inhabitants. The random tests made in Finland during the

CASES OF DIPHTHERIA IN FINLAND IN YEARS 1930—53 AS INFORMED BY THE STATE MEDICAL BOARD AND IN GERMANY IN YEARS 1930—1943 AND 1946—1950, ACCORDING TO KRÖGER AND THOFERN

TABLE 1



epidemic revealed that the disease was caused mainly by *mitis* type and that the toxin-producing strains were relatively rare.

In recent years, clinical diphtheria of severe nature has been extremely rare in Finland, though diphtheria bacilli are still found to some extent in patients with pharyngeal infections. The present work attempts to explain the nature of these cases and to evaluate whether the determination of toxin production is important in estimating the significance of a diphtheria strain as a causative organism of the disease.

MATERIAL AND METHOD

The material was composed of about 8,000 samples studied for diphtheria bacilli in the State Serum Institute during the years 1951—54. *Corynebacterium diphtheriae* was found in 25 persons. The series further included 14 patients from the Aurora Hospital, from whom diphtheria bacilli were isolated. Complete case records for only these 14 patients were at the author's disposal. For the other part of the series the data were based mainly on memory and thus could not be as reliable as the written records. With the excep-

tion of those in the Aurora Hospital, the patients lived in various places in Southern Finland and most of them were cared for by the local physician.

Clauberg II plates containing fluoride were used for isolation of the bacteria (5). The diphtheria types were classified on the basis of their capacity for fermenting glucose, sucrose and starch. The toxin-producing capacity was tested by means of Ouchterlony's plate method. Toxicity of the strains isolated in the Aurora Hospital was determined by the guinea pig test.

RESULTS

On the basis of clinical data, the series was divided into three groups: 1) cases of toxic diphtheria, in which peritonsillar or periglandular oedema, myocarditis or nerve symptoms were the criteria for diagnosis, 2) cases of atoxic diphtheria with throat symptoms and with gray pseudomembranes on the tonsils as the only typical symptom, and 3) cases of symptomless bacillosis. These groups are shown in Tables 2 and 3.

The series included only two cases of clinically toxic diphtheria. Both of them were caused by toxin producing strains, one being of *mitis* and the other of *gravis* type. From the atoxic diphtheria cases listed in Table 2, toxin-producing strains were isolated in four and atoxic strains in eight cases, the corresponding figures for the cases in Table 3 being two and three, respectively. An atoxic strain was isolated in all the bacillosis cases shown in Table 2. From those listed in Table 3, two toxic and seven atoxic strains were isolated.

TABLE 2

CASES FROM WHOM DIPHTHERIA BACILLI WERE ISOLATED IN THE STATE SERUM INSTITUTE IN YEARS 1951—54

Clinical Diagnosis	No. of Cases	Type of Diphtheria Bacilli	Plate Test
Diphtheria toxica	2	gravis	+
		mitis	+
Diphtheria atoxica	12	gravis (2 cases)	+
		mitis (2 cases)	+
		mitis (8 cases)	—
Bacillosis diphtheriae....	11	mitis (10 cases)	—
		intermedius	—

TABLE 3

CASES FROM WHOM DIPHTHERIA BACILLI WERE ISOLATED IN THE AURORA HOSPITAL IN YEARS 1951—54

Clinical Diagnosis	No. of Cases	Type of Diphtheria Bacilli	Guinea Pig Test
Diphtheria toxica	—		
Diphtheria atoxica	5		
of them:			
Diphtheria atoxica ..	(1)	mitis	—
Angina lacunaris.....	(3)	gravis	+
		mitis	+
		?	—
Mononucleosis infectiosa	(1)	mitis	—
Bacillosis diphtheriae....	9	mitis (6 cases)	—
		mitis (1 case)	+
		intermedius	—
		?	+

DISCUSSION

It was observed in the present series that atoxic strains of diphtheria bacilli did not cause clinically toxic diphtheria. Of the clinically atoxic group, toxic strains were isolated in six and atoxic strains in 11 cases. In the bacillosis cases, on the other hand, 18 atoxic and two toxic strains were isolated. The question arises whether we should call all these cases diphtheria or diphtheria bacillosis or whether we should include only those cases in which the bacterial strain has toxin producing capacity? Are the atoxic strains some type of rudimentary or mutation forms of the genuine toxin — producing diphtheria bacilli, or are totally different bacteria involved whose population among the inhabitants is relatively constant during and between diphtheria epidemics? It is apparent from experience that the number of these atoxic strains was almost equal on the basis of the total number of samples, during the epidemic and for some years after it. If atoxic strains represent a rudiment of toxic diphtheria bacilli, it is obvious that the strains rather seldom become toxin-producing again. Atoxic strains would thus be of no epidemiological significance.

Whether atoxic diphtheria bacilli cause certain types of disease and whether they are pathologically important, are different questions. They may cause reddening of the tonsils as well as

pseudomembrane formation, but there may also be entirely different disease-producing agents, such as streptococci, staphylococci, influenza bacilli, *etc.* In the Aurora Hospital, the clinical diagnosis was made independently of the bacterial finding, and only one of the five clinically atoxic cases was diagnosed as diphtheria, three being diagnosed as tonsillitis (in two of which toxin-producing strains were found) and one as typical mononucleosis. Unfortunately the cases in Table 2 cannot be grouped in this way because of lack of data. It is understandable, however, that the local physicians diagnosed the throat inflammation as diphtheria, since diphtheria bacilli were found.

In the present series, *gravis* type was isolated in four cases, and all these strains had toxin-producing capacity. Both of the two strains of *intermedius* type were atoxic. Strains of *mitis* type occurred in 31 cases, five of them being toxin-producing and 26 atoxic.

It is suggested on the basis of the present series that »diphtheria bacilli» which do not have toxin-producing capacity should not be included in the genuine *Corynebacterium diphtheriae* group, but should rather be considered pseudodiphtheria bacilli. The problem is of practical significance especially with regard to diphtheria bacilli of *mitis* type. These strains should always be tested for toxin production, especially when an epidemic is not in question.

SUMMARY

During the years 1951—54, toxin-producing diphtheria bacilli were isolated from only 10 patients. Atoxic strains were found in 29 patients during the same period.

It was noted that atoxic strains did not cause clinically toxic diphtheria. Twenty of these patients were completely symptomless.

Of the toxin-producing strains, two were of *gravis* and the others of *mitis* type. Among the atoxic strains two were of *intermedius* and the others of *mitis* type.

It is suggested that the strains without toxin-producing capacity should not be considered causative organisms of diphtheria and that they should not be given any epidemiological significance.

At least the diphtheria bacilli strains of *mitis* type should always be tested for their toxin-producing capacity, especially when an epidemic is not in question.

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VON DEM KRANKENHAUS DES ROTEN KREUZES IN FINNLAND UND DEM ANATOMISCHEN INSTITUT HELSINKI

ÜBER MEATUS ACUSTICUS INTERNUS

RÖNTGENAUFNAHMEN DES INNEREN GEHÖRGANGES AN 111 SCHÄDELN UND BEI 48 OPERATIV BEHANDELTEN AKUSTIKUSTUMOREN

von

TORSTEN BJÖRKWALL

(Eingegangen am 19. Januar 1955)

Der Meatus acusticus internus ist eine mehr oder weniger trichterförmige Aushöhlung im hinteren oberen Teil des Felsenbeines. Die mediale Öffnung wird Porus acusticus internus genannt. Der Meatus, welcher im Porus am weitesten ist, verschmälert sich etwas nach dem Fundus zu. Da der innere Gehörgang dorso-ventral abgeplattet ist, zeigt das Röntgenbild in verschiedenen Projektionen varierende Form und Grösse. Ventral vom Fundus liegt die Schnecke, während die Bogengänge dorso-lateral gelegen sind.

Durch den Meatus verläuft der N. facialis mit dem N. intermedius, welcher Geschmacksfasern enthält, die durch die Chorda tympani weitergeleitet werden zum vorderen Teil der Zunge. Durch den inneren Gehörgang zieht ausserdem der aus dem N. cochlearis und N. vestibularis bestehende N. acusticus. Durch den Knochenkanal verlaufen weiterhin die Arteria und Vena auditiva interna.

In der Nähe des Porus befinden sich der Hirnstamm mit den nächstgelegenen V—X Kranialnerven, sowie die basalen Hirngefässe. Zwischen den beiden Pori liegt nach vorn zu die Sella turcica, dahinter das Kleinhirn.

Schon vor 50 Jahren sind Röntgenaufnahmen vom Meatus acusticus internus gemacht worden. Die von Schüller (15) angegebene Methode wird in modifizierter Form noch heute bei der Beurteilung von Formvariationen des Meatus angewandt. In diesen 50 Jahren sind zahlreiche Arbeiten über dieses Gebiet erschienen. Die verschiedensten Projektionen sind vorgeschlagen worden. Von diesen komplettiert die schon von Grashey (7) angewandte halbaxiale Projektion am besten die axiale submento-occipitale Aufnahme von Schüller. Später haben Ebenius (6), Lysholm (10) u.a. diese Projektionsmethoden verbessert und vervollständigt. Zusammenfassend sagt Ebenius über seine Untersuchungsergebnisse: »The technique used for examinations, frontal-dorsal and axial symmetrical projections, was theoretically correct and proved to be superior to other available methods». Den Projektionen von Stenvers und ähnlichen erkannte Ebenius nur eine geringe Bedeutung zu. Und zwar, weil man in der Projektion nach Stenvers nur den inneren Gehörgang einer Seite erhält, während die axialen und halbaxialen Projektionen beide Gehörgänge auf einem Bilde darstellen. In der deutschen Literatur werden diese auch submento-vertikal-axiale und Altschul-Uffenorde-halbaxiale Aufnahme genannt (12). Möglicherweise könnte die Methode von Bullo und Dalo (3) für die Untersuchung der Gehörgänge von Nutzen sein.

FRÜHERE UNTERSUCHUNGSERGEBNISSE DES NORMALEN MEATUS ACUSTICUS INTERNUS

Ebenius (6) untersuchte den Meatus acusticus internus röntgenologisch in 100 normalen Fällen und fand hierbei folgende Werte.

TABELLE 1
(NACH EBENIUS)

Projektion	Axial	Halbaxial
Maximum	8 mm	9 mm
Durchschnitt	5.7 mm	6.3 mm
Minimum	4 mm	5 mm

Dieses Material ergab einen Unterschied in der Weite des inneren Gehörganges auf der rechten und linken Seite, wie wir aus der nachfolgenden Tabelle 2 ersehen.

TABELLE 2
(NACH EBENIUS)

Diff. in mm	Axial	Halbaxial
0 mm	62%	67%
1 mm	37%	30%
2 mm	1%	2%
3 mm	0%	1%

Bei einem kleineren Material hat auch LASSILA (9) Messungen des inneren Gehörganges durchgeführt. Seine Ergebnisse stimmen teilweise überein mit denen von Ebenius.

EIGENE UNTERSUCHUNGEN

Um eine genaue Auffassung von der Form und Grösse des inneren Gehörganges zu erhalten, wurde folgende Methode angewandt. Bei 111 von dem Anatomischen Institut Helsinki zur Verfügung gestellten Schädeln wurde der innere Gehörgang mit Eisenfeilspänen gefüllt, indem man die Späne durch einen via Foramen magnun in den Porus geführten Ohrenkatheter rinnen liess. Damit die Späne nicht herausflossen, wurde der Porus mit einem Wachspfropf verschlossen. Nun wurden gemäss Ebenius und Lysholm Aufnahmen in axialen und halbaxialen Projektionen gemacht. Frühere Untersuchungen dieser Art sind dem Verfasser nicht bekannt.

Die so erhaltenen Aufnahmen geben ein scharfes gut begrenztes Bild von Form und Grösse des Meatus. (Fig. 2—9.) Dem in die feinere Röntgendiagnostik des Ohres nicht Eingeweihten erleichtert der röntgendichte Eisenfeilspankontrast die Orientierung und ist somit ein gutes Hilfsmittel beim Studium der anatomischen Details im inneren Ohr, welche auf gewöhnlichen Röntgenaufnahmen oft ziemlich unscharf dargestellt sind. Bei der Beurteilung der gewöhnlichen Röntgenaufnahmen wäre ein solches Modellbild zum vergleich zuempfehlen.

Beim der Betrachtung des Materials zeigt es sich, dass die Form des Meatus in der axialen Projektion trichterförmig ist. (Fig. 2, 3, 4. 5, 10.) Deshalb wäre es falsch den Ausdruck »trichterförmig» in diesem Zusammenhang als pathologisch zu bezeichnen. Beim Verdacht einer pathologischen Formveränderung des Meatus sollet

vorschlagsweise der Ausdruck »pathologisch trichterförmig» angewandt werden.

Zur Bestimmung der Weite des Meatus kann wie bisher entweder die Weite des Porus oder die Weite des Meatus angegeben werden. Wichtig ist nur die Fixation der jeweiligen Art der Messung.

Im vorliegenden Material wurde die Messung des Meatus folgenderweise durchgeführt.

Auf den Bildern in axialer Projektion wird der hintere Rand des Porus bestimmt. Dieser ist gewöhnlich leicht zu finden. Ist das Bild unscharf, zieht man eine Linie von der Spitze der Schnecke im rechten Winkel zur Schneckenbasis und verlängert sie bis zur

Fig. 1. — Das Bild zeigt die gerade Linie gezogen von der Spitze der Schnecke zur hinteren Wand des Porus. Die zweite Linie, welche in der Sagittalebene liegt, bezeichnet die Weite des Meatus. C=N. cochlearis, V=N. vestibularis. Das Bild wird mit der Genehmigung von Prof. F. Henschen publiziert.

hinteren Meatus wand. Vom Schnittpunkt dieser Linie mit der hinteren Wand des Meatus, wird eine weitere Linie in der *Sagittalebene* zur vorderen Meatuswand gezogen. Der Abstand beider Meatuswände im Verlaufe dieser zweiten Linie ergibt nun die Weite des Meatus. (Fig. 1.)

Wird die Weite des Meatus rutinmässig in der obigen Weise angegeben, so dürfte es auch für den nicht Sachverständigen leichter sein, sich auf der Röntgenaufnahme zu orientieren.

Bisher ist zur Bestimmung der Weite des Porus entweder die Weite den Meatus in Stenvers' Projektion oder in halbaxialer Projektion angegeben worden, oder man hat auch in der axialen Aufnahme schräg nach vorn zur abgerundeten Kante gemessen. Besonders die letzte Methode kann der Kritik nicht bestehen. Ausserden sind die Begriffe Porus und Meatus oft verwechselt worden.

Von Bedeutung ist allein das exakte Mass des Meatus beider Seiten. Weiterhin die Struktur des Knochens in der Umgebung des Meatus (6).

Die folgenden Aufnahmen zeigen typische Formen des Meatus acusticus internus, welcher als metalldichter Schatten erscheint. (Fig. 2—11.)

Durch die normale Trichterform des Meatus bedingt, stehen die vordere und hintere Wand in der Axialprojektion in einem gewissen Winkel zueinander. Bei den 222 nneren Gehörgängen des vorliegenden Materials beträgt dieser Winkel im Durchschnitt 21°. (Fig. 4.)

Die Weite des Meatus acusticus internus der 222 untersuchten inneren Gehörgänge veranschaulicht Tabelle 3.

TABELLE 3

DIE WEITE DES MEATUS

Projektion	Axial	Halbaxial
Maximum	8 mm	11 mm
Durchschnitt, rechts	5.4 mm	6.1 mm
links	5.3 mm	6.0 mm
Minimum	3 mm	3 mm

Im gesamten Material beträgt die Differenz zwischen der Weite des rechten und des linken inneren Gehörganges 0.1 mm im Durchschnitt. Tabelle 4 zeigt den genauen Unterschied verteilt auf die einzelnen Fälle.

TABELLE 4

DIFFERENZEN IN MM ZWISCHEN DEN WEITEN DER MEATI

Diff. i mm	Projektionen			
	Axial		Halbaxial	
	Fälle	Prozent	Fälle	Prozent
0 mm	77	69.8	80	72.1
1 mm	29	26.0	30	27.0
	rechts 18		19	
	links 11		11	
2 mm	5	4.5	1	0.9
	rechts 4			
	links 1			

Das Ergebnis stimmt mit den von Ebenius (6) angegebenen Werten nahezu überein. Nach der oben angegebenen Messmethode übersteigt die Differenz der Weite des Meatus beider Seiten nicht 2 mm. Dagegen ist es beachtenswert, dass bei Asymmetrie der rechte Meatus doppelt so oft weiter ist als der linke.

TABELLE 5

DIE WEITE DES MEATUS

Axiale Projektion									
Meatus-Weite	3	4	5	6	7	8	9	10	11 mm
Anzahl Fälle	5	37	87	60	26	7	—	—	—
Prozent	2.2	16.8	39.1	27.1	11.7	3.1			
Halbaxiale Projektion									
Anzahl Fälle	4	19	65	52	48	24	5	3	2
Prozent	1.7	8.5	29.6	23.4	21.6	10.8	2.2	1.3	0.9

Die Tabelle zeigt sowohl die recht varierenden Masse in den verschiedenen Projektionen, als auch, dass die Mehrzahl der Meati eine Weite von 5 mm ergibt. In der gleichen Anzahl der Fälle mit 5 mm Meatusweite trifft man in der axialen Projektion Weiten von 6 und 7 mm. Auf Grund der in dorso-ventraler Richtung abgeplatteten Form des Meatus sind sämtliche Masse in der halbaxialen Projektion grösser, so dass hier 8 mm keine Besonderheit darstellt. Selten trifft man einen normalen Meatus mit 3 mm oder grösseren Weiten als 9 mm.

TABELLE 6

DIE LÄNGE DES METAUS

Projektion	Axial	Halbaxial
Rechts.....	8.2 mm	8.9 mm
Links	8.1 mm	8.6 mm
Durchschnitt	8.5 mm	

Die Länge des Meatus acusticus internus ist wie die Tabelle 6 zeigt im Durchschnitt 8.5 mm.

FRÜHERE UNTERSUCHUNGEN DES MEATUS ACUSTICUS INTERNUS BEI AKUSTIKUSTUMOREN

Henschen (8) hat erstmalig einen Akustikustumor im Röntgenbild nachgewiesen. An Hand einer Reihe wertvoller Arbeiten hat er die Entstehung der Akustikustumoren geklärt. Der Tumor entwickelt sich in dem distalen Abschnitte des N. vestibularis auf dem Boden des Meatus. Diese Beobachtung dürfte von Bedeutung sein bei der Beurteilung der von Brunner (2) angegebenen gestielten medialen Akustikustumoren. Bei der Beurteilung von Röntgenbildern ist dies von Wichtigkeit.

In den lezten 50 Jahren ist viel über Akustikustumoren geschrieben worden. Verschiedene Autoren haben sich mit der Technik der röntgenologischen Darstellung und deren Auswertung befasst. Die wertvollsten dieser Arbeiten sind bereits oben erwähnt worden. Ebenius (6) legt bei der Beurteilung dieser Tumoren besonders Gewicht auf gewisse Details im Röntgenbild. Diese sind: »Expansion und Deformierung des Meatus acusticus internus, Decalcifikation bei fehlender Erosion des Apex, Decalcifikation mit Erosion des Apex oder des Dorsum sellae, Destruktion und Atrophie des Dorsum sellae und Clivus und schliesslich Decalcifikation in der Umgebung des Foramen magnum oder andere Veränderungen des Schädels als Zeichen erhöhten Hirndruckes». Hinsichtlich der Symptomatologie kann auf die Standardarbeiten auf diesem Gebiet hingewiesen worden (1, 4, 5, 14).

Eine Zusammenfassung der Erfahrungen an 300 operierten Akustikustumoren hat Lundborg (11) publiziert. Von den 296 röntgenologisch explorierten Fällen zeigten 15.2 Prozent keinerlei wahrnehmbare Veränderungen, obgleich sich darunter 18 sehr grosse, 22 grosse und 5 kleine chirurgisch nachgewiesene Tumoren befanden. In 161 Fällen handelt es sich um rechtsseitige, in 139 Fällen um linksseitige Tumoren. In einer Übersicht über 3 andere Untersuchungsmateriale hat man in nur ca der Hälfte der Fälle den Tumor röntgenologisch diagnostizieren können. Es handelt sich dabei um ein grosses Krankenmaterial, welches je 175,42 und 145 operierte Akustikustumoren umfasst.

Die von Ebenius beschriebenen normalen Kontrollfälle weisen in 59 Prozent einen Unterschied in der Weite des Meatus der rechten und der linken Seite auf. Von 34 radiologisch untersuchten operier-

ten Tumorfällen fand er in 5 Fällen keine pathologische Veränderung der Schädelbasis. Bailey (1) und Dandy (5) äussern sich skeptisch gegen die Bedeutung der Röntgenuntersuchung für die Diagnose der Akustikustumoren. In seinem 95 operierte Fälle umfassenden Material hat Nylén (13) in 64.4 Prozent röntgenologisch einen dilaterierten Porus feststellen können. Neunzig Prozent zeigten röntgenologische Veränderungen der Schädelbasis.

EIGENE UNTERSUCHUNGEN

Das Material umfasst 48 Fälle operierter Akustikustumoren, welche sämtlich vor der Operation einer genauen Röntgenuntersuchung unterzogen worden sind. Angewandt wurde die von Ebenius (6) und Lysholm (10) angegebene Methode. Die Operationen wurden im Krankenhaus des Roten Kreuzes zu Helsinki ausgeführt.

In 42 Fällen konnte der Tumor vor der Operation röntgenologisch diagnostiziert werden, die übrigen 6 Fälle zeigten in der Röntgenaufnahme keine pathologische Veränderung der Schädelbasis.

TABELLE 7

MEATUS-WEITE BEI AKUSTIKUSTUMOREN

	Nach dem Verfasser		Übliche Mess. method	
	axial	halbaxial	axial	halbaxial
Meatus-Weite im Durchschnitt ..	9 mm	8.6 mm	12.4 mm	12.4 mm
Maxim. Diff. zw. rechts u. links ..	14 mm	17 mm	19 mm	18 mm
Min. Diff. zw. rechts u. links	2 mm	1 mm	2 mm	2 mm
Durchschnitt von Diff. rechts u. links	5.4 mm	3.9 mm	6.3 mm	7 mm

Wie aus den obigen Ziffern hervorgeht, (Tab. 7.) sind die Resultate der Messungen des Meatus acusticus internus bei beiden Messverfahren verschieden. Die obige Tabelle umfasst 48 Fälle mit operativ festgestelltem Akustikustumor. Wie ersichtlich ist der Durchschnitt der Meatusweite in mm bedeutend niedriger nach der vom Verfasser angegebenen Methode, als bei den üblichen Methoden. Auch der Durchschnitt der Unterschiede zwischen rechts und links sind bedeutend; dies gilt auch für die halbaxiale Projektion.

Nach diesem Material wird in der Regel ein Akustikustumor erst bei einem röntgenologisch messbaren Unterschied von ca 3—4 mm zwischen dem rechten und linken Meatus diagnostiziert.

Auch in diesen Fällen mit Akustikustumoren ist der durch die Trichterform bedingte Winkel gemessen worden und ergab einen Durchschnittswert von 51°. Zum Vergleich sei der normale Wert von 21° erwähnt.

Obgleich das Kontrollmaterial dieser Arbeit einem Anatomischen Institut entnommen ist, zeigt es eine geringere Differenz im Unterschied zwischen der Weite des rechten und linken Meatus acusticus internus wie das lebende Material von Ebenius. Eventuell könnten höhere Durchschnittswerte, vergleichen mit dem Material von Ebenius, durch das Fehlen des Periosts bei dem zur Verfügung gestandenen Material bedingt sein. Die tatsächlichen Werte sind dagegen um einige Zehntel mm geringer. Nach Ansicht des Verfassers ist dies durch die zuverlässigere Messungsmethode bedingt.

ZUSAMMENFASSUNG

An 111 Schädeln ist der Meatus acusticus internus auf beiden Seiten mit Eisenfeilspänen gefüllt worden. Von diesen Schädeln wurden dann Röntgenaufnahmen in axialer und halbaxialer Projektion gemacht. Nach einem bestimmten Schema wurden Messungen der Weite des Meatus an gleichen Stellen vorgenommen. Die Messungen geschahen in der Sagittalebene. Es wird vorgeschlagen, den Ausdruck »trichterförmig» durch »pathologisch trichterförmig» zu ersetzen. Die normale Trichterform des Meatus bildet einen Winkel von 21°. Die normale Weite des Meatus in axialer Projektion beträgt 5.3—5.4 mm und in halbaxialer Projektion 6.0—6.1 mm. Der rechte Meatus ist normal 0.1 mm breiter als der linke. Der Meatus ist gewöhnlich 8.5 mm lang.

Bei 48 Akustikustumoren war der Meatus gewöhnlich in beiden Projektionen 8—9 mm weit. Im allgemeinen wurde der Tumor erst bei dieser Weite diagnostiziert. Auf beiden Seiten war die maximale Differenz bis zu 19 mm und die minimale bis zu 1 mm gefunden worden. Bei diesen Akustikustumoren war der durch die Trichterform bedingte Winkel des Meatus im Durchschnitt 51°.

SUMMARY

On 111 skulls Meatus acusticus iternus has in both sides been filled with iron filings. The skulls have been X-rayed in axial and half-axial projection. The width of meatus has always been measured in the same place in the picture following a certain system. The measure of meatus has been done in the sagittal-plane. The expression »pathological funnel-formed» should be used in stead of the expression »funnel-formed». The normal funnelform forms an angle of 21 degrees of meatus. The width of meatus is normally in the axial projection 5.3—5.4 mm and in the half-axial projection 6.0—6.1 mm. The right side is normally 0.1 mm wider than the left side. The meatus is 8.5 mm long.

In 48 cases of acoustic tumors the meatus was usually 8—9 mm wide in both the projections. The acoustic tumors were usually not diagnosticated until the meatus had reached this width. Maximal differences of 19 mm and minimal differences of 1 mm between the right and the left side have been measured. The average degree of the funnel-form of the meatus in these 48 cases of acoustic tumors is 51 degrees.

CONCENTRATO

Sulle due parti di 111 crani si é riempito il meatus acusticus internus con limatura di ferro e dopo si é fatta la radiografia in proiezione assiale e semiassiale. La larghezza del meatus si misura sempre nelló stesso punto secondo una schema prestabilito. La misurazione del meatus si fa sul piano sagittale. L'espressione »a forma d'imbuto» deve essere sostituita con l'espressione »patologico a forma d'imbuto». La forma d'imbuto normale ha un angolo di 21°. In proiezione assiale la larghezza normale del meatus é di 5.3—5.4 mm. In proiezione semiassiale la larghezza é 6.0—6.1 mm. La parte destra é 0.1 mm piú larga della sinistra. La larghezza nei piu grandi meati misurava nel materiale normale (222 meati) 8 mm nella proiezione assiale e 11 mm nella proiezione semiassiale. I piú piccoli misuravano 3 mm in tutte e due le proiezioni. La lunghezza del meatus normale é di 8.5 mm.

Si é constatato una differenza sensibile nelle misurazioni fatte con i sistemi tradizionali rispetto a quelle fatte dall'autore sulla

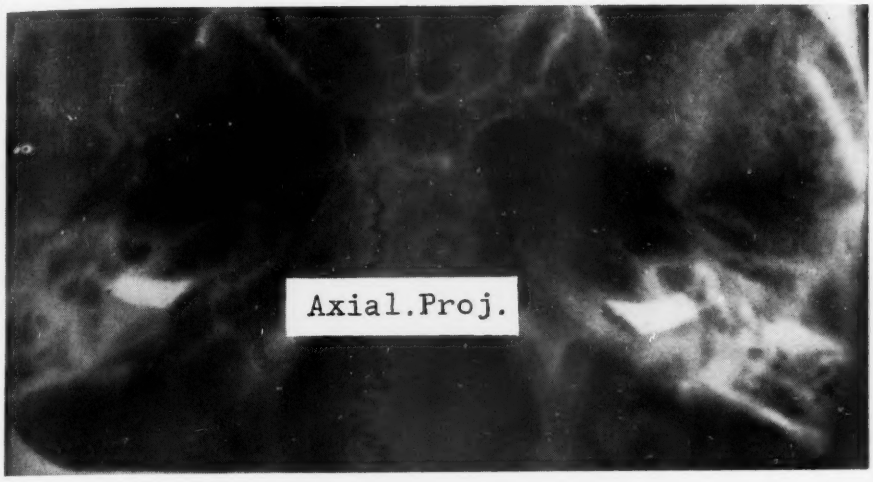


Fig. 2.

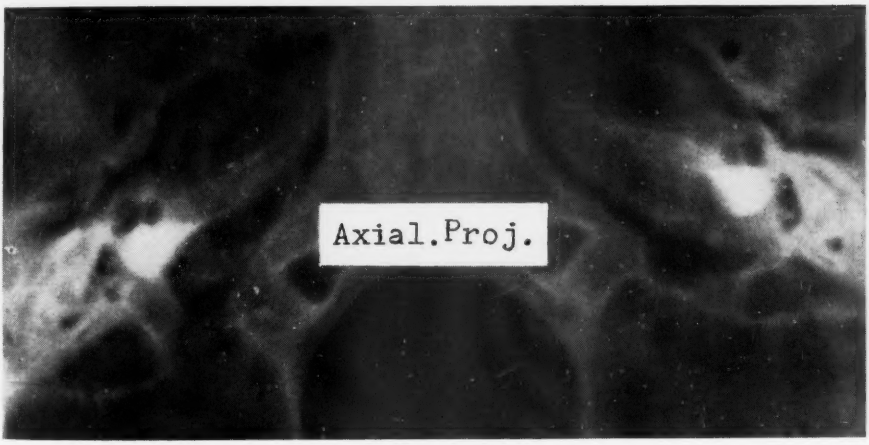


Fig. 3.

Fig. 2—11. — Die Röntgenaufnahmen zeigen normale Meati von verschiedenen Formen.

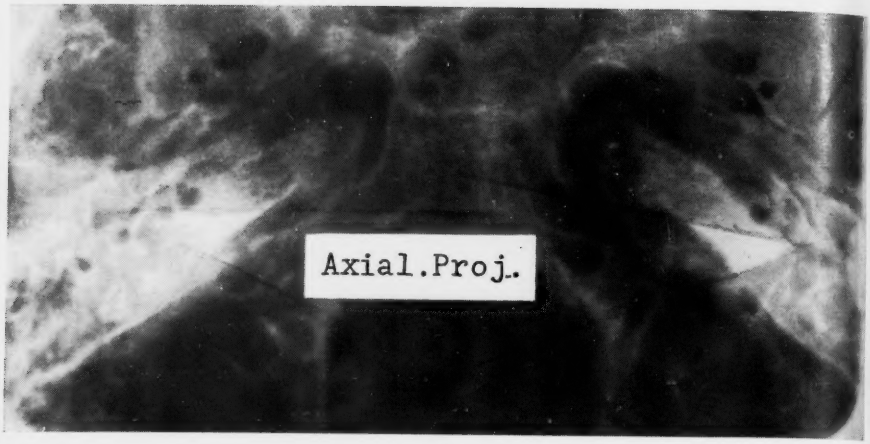


Fig. 4.

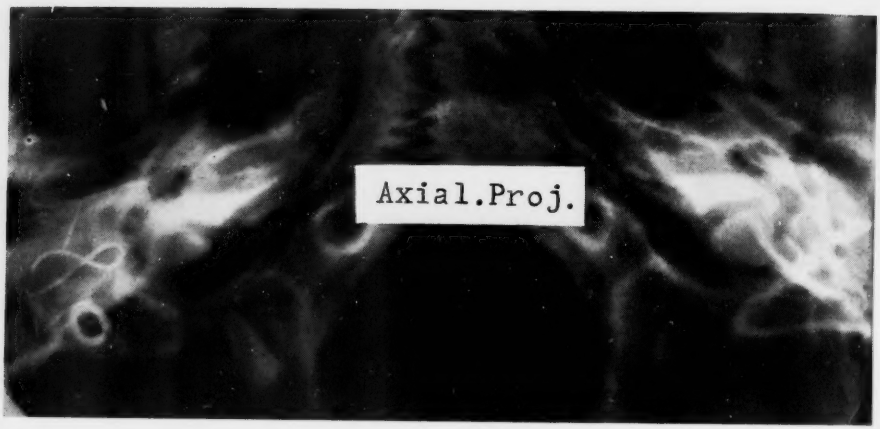


Fig. 5.

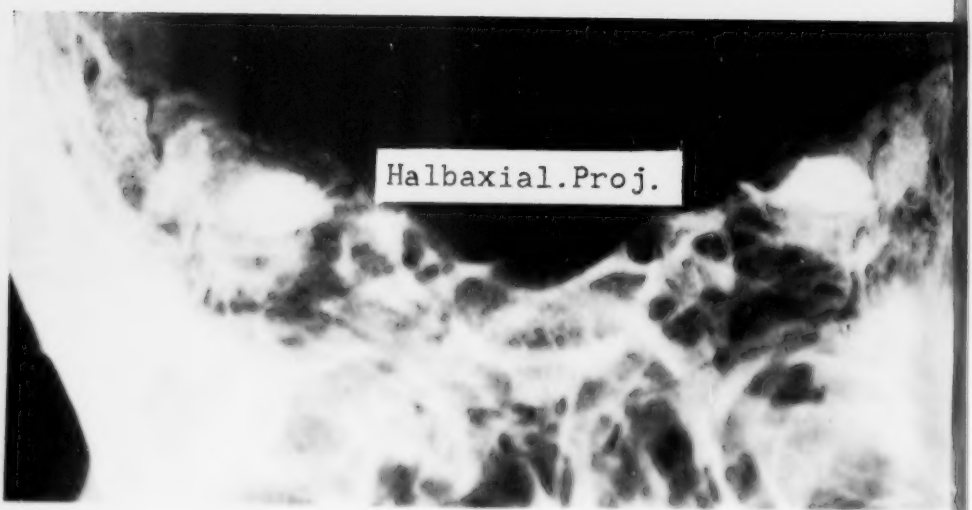


Fig. 6.

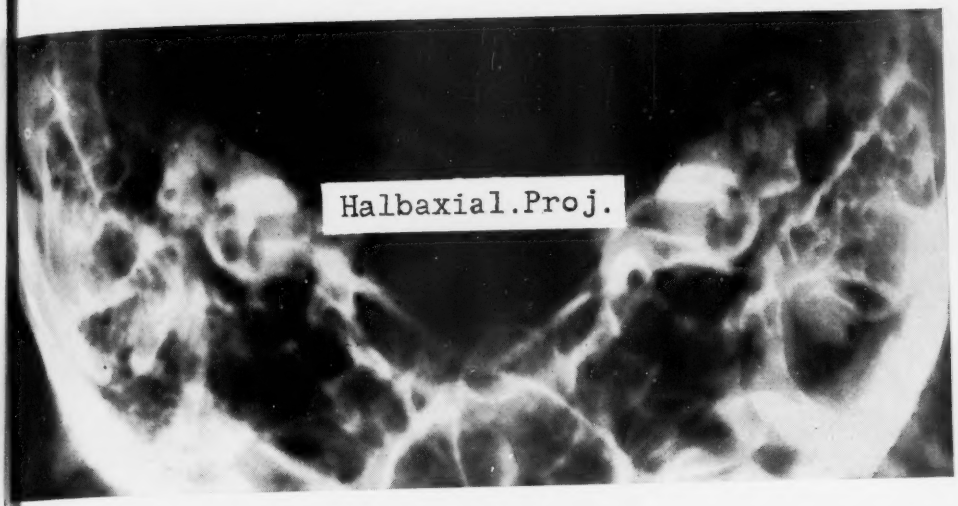


Fig. 7.

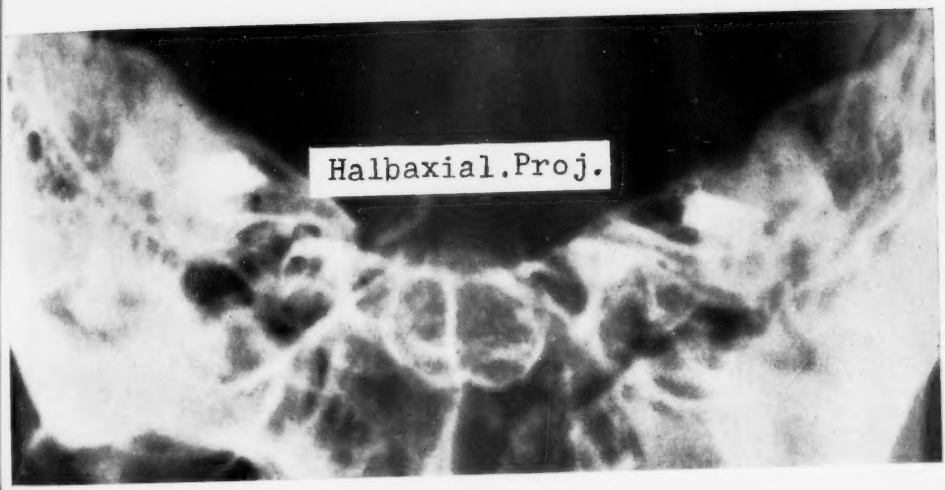
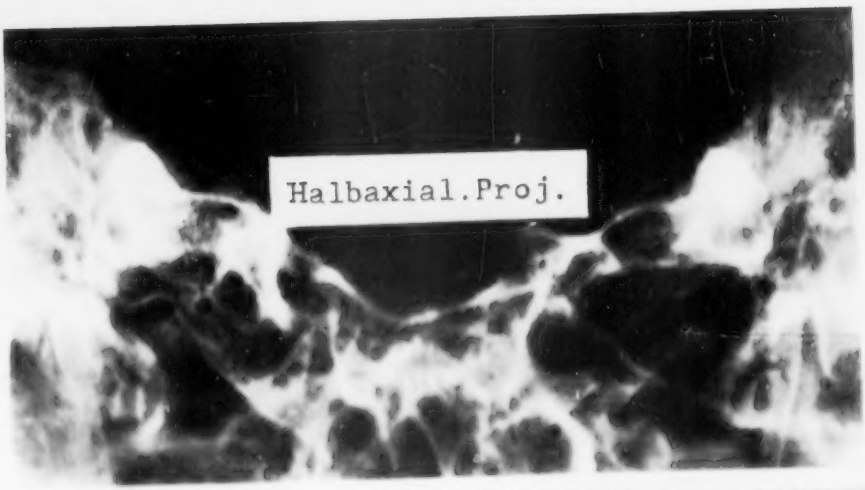


Fig. 8.



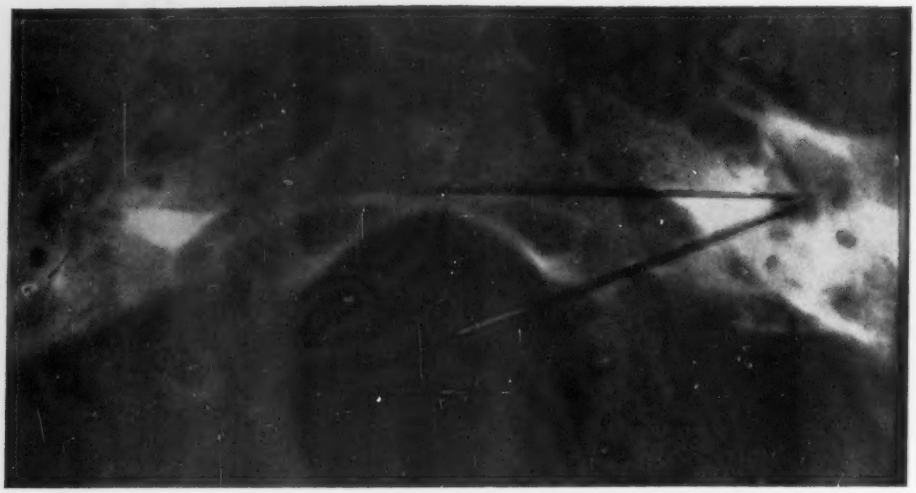


Fig. 10. — Die normale Trichterform des Meatus.

Fig. 11.

base di 48 casi di tumori acustici. Il valore medio della larghezza del meatus in un tumore acustico é di 8.6 mm in proiezione semiassiale e di 9 mm in proiezione assiale. Nel caso di tumori acustici la forma d'imbuto ha un angolo di circa 51°.

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BENTONITE PURIFICATION OF CERTAIN VIRUSES[1, 2]

by

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The most commonly used physical and chemical methods of purifying viruses for different purposes have been reviewed in the modern textbooks of virology (3, 17). The use of ion-exchange resins for this purpose has been discussed in several recent publications (8, 9, 10, 18).

In a previous paper we described a method involving the use of Bentonite for the partial purification of a Type A strain of the Coxsackie group of viruses from the brain-muscle tissue of suckling mice (13). The Bentonites belong to the clay minerals and some of them are known for their high ion-exchange and adsorbing capacities (4, 5, 6). According to our experiments on the effect of Bentonite on human serum, especially lipids and/or lipo-proteins are adsorbed (11, 12). In the experiments with the Coxsackie virus it was shown that a simple one step precipitation with one per cent Bentonite resulted in the adsorption of all the cholesterol, 85 per cent of the phospholipids and about 50 per cent of the total nitrogen of the tissue extract without any decrease in the titer of the virus. The preparations obtained, colorless and water clear, gave good results

[1] This investigation was aided by grants from the Sigrid Jusélius' Foundation.

[2] A preliminary report on the results presented in this paper was read at the International Congress of Microbiology in Rome, Sept. 6—12, 1953.

[3] With the technical assistance of Miss Irja Rouhia, to whom the authors wish to express their sincere thanks.

as antigens in the complement fixation test. Comparison between this antigen and antigens prepared either by protamine sulphate precipitation (7, 19) or purified by the acetone — ether extraction method (2) showed that at least the same degree of purity was obtained.

Because of the simplicity of the method attempts were therefore made to purify certain other viruses too by this method. This paper deals with the results obtained with Bentonite purification of Coxsackie viruses of group A and B, the Lansing strain of poliomyelitis, the Rabies virus and the Rous sarcoma agent.

MATERIAL AND METHODS

Viruses

Coxsackie Viruses. — Two strains of group A Type 10 were used, one of them obtained as a type strain from Professor Sven Gard, Stockholm. The other strain was isolated in Finland during an epidemic of poliomyelitis in 1950 (14). The strains were passed in suckling mice one to four days of age by injecting intramuscularly 0.02—0.03 ml of a 20 per cent brain-muscle extract from infected mice. The mice were sacrificed as soon as paralysis occurred, usually after 3—4 days.

The Coxsackie B strain used was a type B1 strain, also obtained from Professor Gard. Passages were made in suckling mice one to four days of age, injected intracerebrally with 0.02—0.03 ml of a 20 per cent brain suspension of infected mice. The mice were sacrificed on the fifth day after infection, when typical symptoms occurred.

Titrations of both the Coxsackie viruses were performed by using one to two litters of suckling mice, each containing 6 to 15 animals, for each ten-fold dilution.

The Lansing Strain of Poliomyelitis. — This strain, supplied by Dr. K. Penttinen, Helsinki, was obtained from the Virus Laboratory, Ann Arbor, Michigan, U.S.A. The strain was in its 81st mouse passage. Passages were made in young mice weighing 12 to 15 grams each by intracerebral injection of 0.02 to 0.03 ml of a 20 per cent suspension of infected mouse brain. The mice were sacrificed as soon as paralysis occurred in one or several of the mice in the group

infected, on an average after 7 to 8 days. Titrations were performed with groups of 10 mice for each ten-fold dilution.

The Rabies Virus. — The strain used was the egg-adapted Flury strain, obtained after one mouse brain passage from Professor Gard. It was passed intracerebrally in young mice in the same manner as the Lansing virus. The mice were usually sacrificed on the 8th to 9th day after infection. In the titrations, ten mice were used for each ten-fold dilution.

The Rous Sarcoma Virus. — The Rous sarcoma was obtained from Dr. F. Duran-Reynals, Yale University, New Haven, Conn., U.S.A. in 1950 and has since then been kept in alternate egg and chicken passages in this laboratory. The tumors were produced in one to two weeks old Leghorn chickens which were injected intracutaneously, subcutaneously and intramuscularly with 0.5 ml of a 20 per cent tumor suspension. The chickens were sacrificed after about two weeks, when the tumours as a rule were about 5 cm in diameter. Titrations were performed in chickens of the same age by using ten-fold dilutions, three to four chickens for each dilution.

Tissue Extracts. — 20 per cent tissue extracts were used as starting material for titrations and purification. The brain-muscle tissue was minced in a Waring Blendor and the brain and sarcoma tissue by grinding the tissue in a mortar with or without sterile sand. The suspensions were made up in a phosphate buffer, pH 7.2, containing 50 units of penicillin and 0.05 mg of streptomycin per ml. The brain muscle tissue was kept at +4° C overnight and then centrifuged at 15.000 g for 15 minutes in an International PR1 Angle Centrifuge. The brain and the sarcoma suspensions were centrifuged immediately in an angle centrifuge at 1000 g for 15 minutes.

Bentonite. — The brand of Bentonite used for these experiments was Meyenita BG 200, obtained through the courtesy of Dr. Y. Megias, Instituto L'lorrente, Madrid, Spain. From the greyish powder a 2 per cent aqueous suspension was made. The suspension was sterilized in an autoclave at 120° C for half an hour, and stored in an ice box. When flocculation occurred in the suspension it was discarded.

Precipitation with Bentonite. — To the 20 per cent tissue extracts were added equal parts of 2 per cent Bentonite suspension. The mixture was carefully shaken and left to stand at room tempera-

ture for 30 minutes. The heavy precipitate formed was spun down in an angle centrifuge at 1000 g for 15 minutes, and the usually colorless and water clear supernatant was collected. It represented the partially purified preparations. These preparations were tested for infectivity in the same manner as described for each virus.

Eluation Experiments. — In the cases where attempts were made to eluate the virus from the Bentonite sediment, the sediment was carefully re-suspended in either a borax buffer of pH 9.0 or in 5 per cent sodium chloride solution. After one hour at room temperature or 24 hours at 4° C the suspension was again centrifuged as above and the supernatant tested for infectivity.

Precipitation with Protamine Sulfate. — The method of Kraft and Melnick (7) for the preparation of protamine-treated crude antigen was used in preparing the protamine antigen of the Coxsackie virus.

Immunisation (Coxsackie Virus). — Young mice were injected twice a week intraperitoneally with 0.2 ml of the protamine-treated or Bentonite-treated virus suspension diluted first 1: 100 and later 1: 10. Six injections were given altogether. One week later the mice were bled by heart puncture, the serum was collected, diluted 1: 2 with phosphate buffer, and inactivated at 56° C for 30 minutes.

Neutralisation (Coxsackie Virus). — Serial two-fold dilutions of the immune sera were thoroughly mixed with 2×100 ID_{50} of the virus and left to stand at room temperature for one hour. Of the virus serum mixture 0.02 to 0.03 ml was then injected either intramuscularly or intracerebrally into suckling mice two to five days of age.

Complement Fixation Test (Coxsackie Virus). — The complement fixation tests were carried out according to a method described earlier (14).

Storage. — All the virus suspensions as well as the infected tissues and the sera were, unless otherwise stated, stored in a deep freeze at —15° C to —20° C. The albumin used for some of the experiments was Difcos Dubos Medium Albumin.

Calculation of Endpoints. — The titration endpoints were calculated by the method described by Reed and Muench (16).

RESULTS

As has already previously been shown (13), the Coxsackie virus of group A could easily be partially purified by Bentonite. The present experiments showed the same to be true also of the Coxsackie virus of group B and the Lansing strain of poliomyelitis. In all the experiments performed with these two viruses the partially purified supernatant always had a virus titer of the same magnitude as the original tissue extract. In the case of the rabies virus, however, all the experiments showed a clear decrease in the virus titer of the Bentonite-treated supernatant compared with the original extract. This was still more markedly the case with the Rous sarcoma virus (Table 1).

The table includes the values of the total nitrogen per ml of extract and partially purified supernatant. With the Coxsackie viruses, the Lansing virus and the rabies virus the total nitrogen decreases to about one third its amount in the original extract. In the case of the Rous sarcoma virus the decrease in total nitrogen is about five-fold. All or practically all the cholesterol and 30 to 85 per cent of the phospholipids are adsorbed to the Bentonite sediment.

Because of the marked decrease in the titer of the rabies virus and the Rous sarcoma virus it was assumed that the virus was probably precipitated along with other tissue material in the Bentonite sediment. Attempts were therefore made to free the virus from the bulk of the sediment by eluation. With the rabies virus, eluation with sodium chloride or with borax buffer resulted in apparently fairly pure preparations containing, however, only about one tenth of the amount of virus assumed to be in the sediment.

The eluation experiments with the Rous sarcoma virus were still less succesful; the eluates had virus titers of only 10^{-1} and 10^{-2}, respectively, compared with a titer of 10^{-5} in the original extracts.

The temperature, pH and ionic strength, however, were not very well controlled, and it is therefore difficult to state if the results depend purely on inactivation of the virus or on inadequate eluation methods. It may be pointed out, however, that the virus treated with Bentonite behaved, as far as incubation period and macro-

TABLE 1

VIRUS TITER AND NITROGEN CONTENT OF CERTAIN VIRUSES UNPURIFIED AND PARTIALLY PURIFIED WITH BENTONITE

Type of Virus	Material Tested	Virus titer of				mg N/ml of			
		Tissue Extract	Bentonite Supernatant	NaCl Eluate	Borax Eluate	Tissue Extract	Bentonite Supernatant	NaCl Eluate	Borax Eluate
Coxsackie A	mouse muscle and brain	7.7	7.6			0.85	0.24		
Coxsackie B	mouse brain	5.2	5.4			1.02	0.39		
Lansing	mouse brain	2.3	2.5			0.82	0.23		
Rabies	mouse brain	4.5	3.0	3.6	3.4	0.77	0.21	0.06	0.1
Rous sarcoma	sarcoma tissue	5.2	2.5	2.0	1.0	0.45	0.09	0.03	0.03

TABLE 2

COMPARISON BETWEEN COMPLEMENT FIXATION TESTS AND NEUTRALISATION TESTS OF TWO COXSACKIE A-IMMUNE SERA

Serum	Type of Antigen	Complement Fixation Test								Neutralisation Test				
		Serum Dilution								Serum Dilution				
		8	16	32	64	128	256	512	1024	50	100	200	400	800
Immunisation with Bentonitetreated virus	Bentonitetreated	4*	4	4	4	2	2	0	0	0/8**	0/10	0/12	9/10	17/17
	Protaminetreated	4	4	4	4	1	0	0	0					
Immunisation with Protaminetreated virus	Bentonitetreated	4	4	4	4	2	0	0	0	0/11	0/12	0/9	12/12	9/9
	Protaminetreated	4	4	4	4	1	0	0	0					

* Degree of hemolysis inhibition
4 = total inhibition
2 = 50 per cent inhibition
0 = no inhibition

** Denominator = number of mice injected
Numerator = Number of mice dead

scopical symptoms in the animals are concerned, in all respects similarly to untreated virus.

In this connection it may also be mentioned that the Lansing virus showed a very marked irregularity in the incubation period. This varied from 6—7 to as much as 13—15 days, and sometimes to 20 days. Similar results have recently been mentioned by Ainslie (1). A somewhat similar although less pronounced phenomenon was observed in the experiments with the rabies virus.

TABLE 3

COMPARISON BETWEEN THE STABILITY OF UNPURIFIED VIRUS AND VIRUS PARTIALLY PURIFIED BY BENTONITE

Virus	Material Tested	Virus Titer				
		Before Storage	after Storage at —15° C for			
			1 Month	3 Months	12 Months	22 Months
Coxsackie A	Tissue extract	7.7	7.8	7.6	7.5	7.7
	Bentonite supernatant	7.6	7.3	6.8		6.6
	Bentonite supernatant + 1 per cent albumin	7.6	7.5	7.3		7.3
Coxsackie B	Tissue extract	5.2	5.1			5.2
	Bentonite supernatant	5.4	5.0			
	Bentonite supernatant + 1 per cent albumin	5.2				5.1

Experiments concerned with the properties and the stability of the Bentonite-purified virus preparations were performed only with the Coxsackie viruses. It was shown in several experiments that the immunizing properties of the Bentonite-treated virus were the same as those of untreated virus or virus purified with protamine sulfate. Bentonite-treated virus was also compared with protamine sulfate-treated virus in the complement fixation test. There was no marked anti-complementary action of the preparation and the results obtained concurred well to those obtained with protamine-purified crude antigens. In Table 2 are shown the results with two mouse immune sera tested with both Bentonite-treated virus and protamine sulfate-treated virus in the complement fixation test and compared with simultaneously performed neutralisation tests.

The usefulness of Bentonite-treated Coxsackie virus for the testing of complement fixing antibodies in human sera has been shown by Pohjanpelto (15) with many of the known group A types of Coxsackie virus.

The stability of the Bentonite-treated preparations was tested by titrating preparations stored in the deep freeze at —15° C to

—20° for varying length of time (Table 3). It was shown that one month of storage did not affect the titer of the virus. Three months of storage, however, brought about a clear decrease in the titer of the Coxsackie A virus. The Coxsackie B virus was not tested after this storage period. When the preparations were tested after 22 months of storage there was a marked decrease in the titer of the partially purified preparations, but practically no change in that of the original extracts. Addition of one per cent albumin to the partially purified preparations resulted in preparations as stable as the original extract.

DISCUSSION

From the experiments presented here it appears that adsorption with Bentonite is a simple and apparently well suited method for the partial purification of the Coxsackie group of viruses and the Lansing strain of poliomyelitis virus from lipid-containing tissue material. Bentonite adsorbs most of the cholesterol and about two third of both, the phospholipids and the total nitrogen, leaving the virus intact in the supernatant.

The other two types of viruses tested, viz. the rabies virus and the Rous sarcoma virus, under the experimental conditions used, could not be purified as easily. The experiments performed to eluate the virus from the bulk of the Bentonite sediment showed that only small amounts of virus could be recovered. Whether this is due to inactivation of the virus or to inadequate methods remains obscure.

Bentonite is a cation-exchanger. The principle is therefore probably the same as in using different ion-exchange resins for virus purification, which has been done for instance with influenza virus and some neurotropic viruses (8, 9, 10, 18). Depending on the type of virus and the ion-exchange resin used, the virus is either adsorbed into the resin or remains in the supernatant fluid. The Lansing virus, for instance, was adsorbed to an anion-exchange resin (8) whereas some encephalitis viruses were not adsorbed to a cation-exchange resin (9). In the cases where the virus is adsorbed it is usually easily eluated from the ion-exchange resin. It was seen, however, that this is not the case with Rabies virus and the Rous sarcoma virus in these experiments.

It seems reasonable to assume that some of the other neurotropic viruses may behave in the same manner as the Coxsackie viruses and the Lansing virus, in which case Bentonite precipitation may prove a practical tool for their partial purification.

The inexpensiveness of Bentonite and the simplicity of the method make it useful for the production of suitable antigens for the complement fixation tests with, for instance, the Coxsackie group. It might also prove of value, in some cases, for a preliminary purification of viruses from lipid-containing tissue material for further studies of the properties of certain viruses.

SUMMARY

A method for the partial purification of the Coxsackie group of viruses and the Lansing strain of poliomyelitis from mouse brain is described. The method involves the use of Bentonite adsorption of non-virus material, especially lipids, from tissue extracts.

Coxsackie virus purified by this method proved suitable as antigen in the complement fixation test.

Such partially purified preparations are stable for one to two months at —15° C, and after the addition of one per cent albumin for at least 22 months.

Attempts to purify a strain of rabies virus and the Rous sarcoma virus by the same method resulted in a marked decrease of virus in the »lipid-free» supernatant. The virus could only be recovered to a small extent from the bentonite sediment.

The probable mechanism of the method and its applications are briefly discussed.

We wish to thank Professor Sven Gard, Dr. F. Duran-Reynals and Dr. K. Penttinen who have kindly supplied most of the virus strains used for this experiments, and Dr. Y. Megias for supplying the Bentonite used.

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FROM THE MEDICAL DEPARTMENT OF THE KIVELÄ HOSPITAL, HELSINKI

SERUM CONCENTRATIONS OF PENICILLIN IN GERIATRIC PATIENTS

by

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(Received for publication January 25, 1955)

What happens in the human organism to the penicillin administered by different routes is known in detail. This information, apart from its necessity for the clinical use of penicillin, can be applied to the study of numerous physiological and pathological problems. The differences due to age in the serum concentrations of the different types of penicillin have been discussed e.g. by Seeler et al (5), Gockell and Sprenger (2), and Weingärtner (7), who found penicillin blood levels to rise with advancing age.

The object of the present investigation was to study the penicillin metabolism of geriatric patients proper and compare it with that of young test subjects. Two groups of nine persons each were used in the experiments: persons over 70 and persons of about 30 or under. Efforts were made to select from the hospital material subjects whose disease does not, as far as is known, affect penicillin concentrations. No manifest signs of renal insufficiency were found in the test subjects, they had no fever, and they had not been given antibiotics in the period immediately preceding the experiment.

Each patient was given 200,000 units of crystalline G-penicillin (Leopenicillin, Medical Suppliers Leo) dissolved in 2 ml of physiological saline solution. The injection was made deep into the gluteal region. The serum samples were taken ½, 1½, 3 and 6 hours after the injections, centrifuged, and their penicillin titre determined by a modified (3) WHO Sarcina lutea Cup-Plate method (6). The

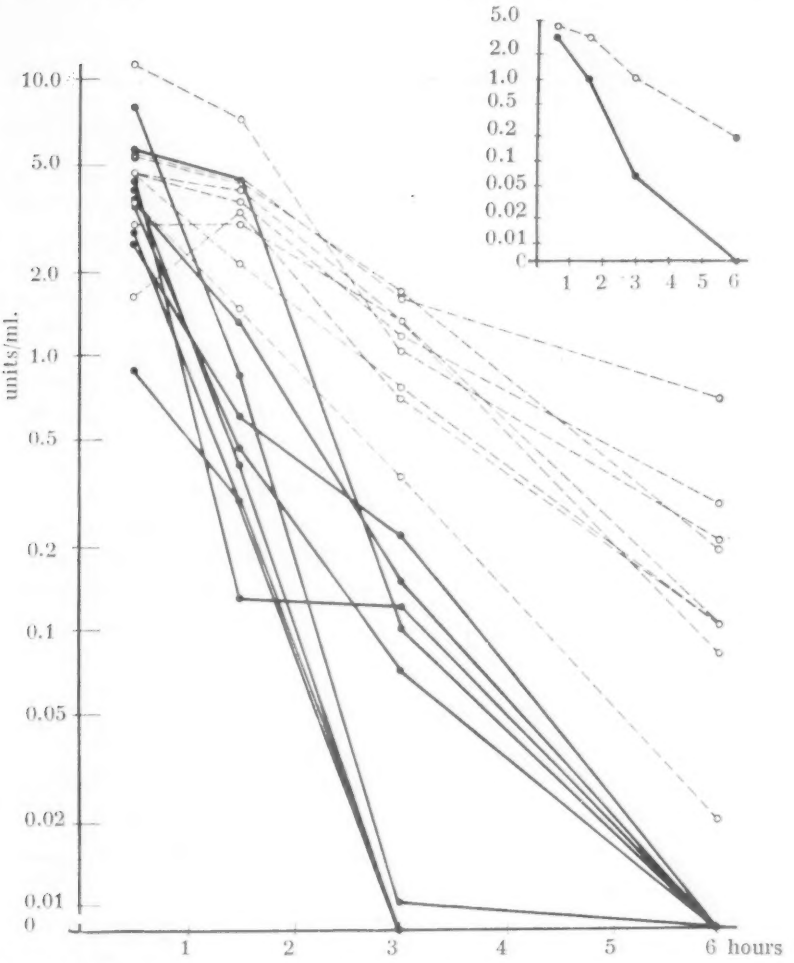

Fig. 1. — Serum concentrations of 9 geriatric patients — — — and 9 control subjects ———. Inset graph gives the corresponding means. Logarithmic scale used in both.

patients remained in bed during the test but no attention was paid to their consumption of fluid and food. Urine secreted during six hours (catheterized if necessary) was collected and mixed, and its penicillin concentration was determined by the same method.*

The serum concentrations of the patients are shown in Fig. 1 and the results of the urine examinations and the patient's age and

*The determinations were made by Mrs. Helvi Vilppula, M.Sc., of the microbiological laboratory of Medical Suppliers Orion.

TABLE 1

URINE EXAMINATIONS OF AND DATA ON THE PATIENTS, AND THEIR MEANS.

Diagnosis	Age	Weight, kg	Urine		
			Amount, ml	Penic., Units/ml	Total Amount of Penic., Units
Myodegen. cordis	83				
Apoplexia	79				
»	86	57.6	250	150	37,500
»	86	47.8	120	115	13,800
»	75	59.4	500	135	67,500
Thrombosis art. cerebri?	73	47.4	300		
Hypertonia	73	60.0	150	350	52,500
Carcinoma prostatae ..	78	76.0	200	70	14,000
Marasmus senilis	85	67.0	200	175	35,000
Geriatric patients, means	79.8	59.3	246	166	36,717
Nihil	29	82.0			
»	33	80.0			
Psychoneurosis	26	84.0			
Anemia post infectionem	29	55.0	210	100	21,000
Asthma bronchiale ..	24	61.5	320	175	56,000
Cholecystopathia?	23	72.3	300		
Ulcus duodeni	29	67.5	415	200	83,000
» »	30	61.0	400	90	36,000
» »	30	53.8	520	55	28,600
Control patients, means	28.1	68.6	361	124	44,920

weight in Table 1. Serum samples taken before penicillin injection contained no substances inhibiting the growth of Sarcina lutea. Samples taken ½ hour after penicillin injection showed no appreciable difference. After 1½ hours the titres of the control group, with one exception, were lower, after 3 hours all without exception. The 3-hour means were 0.074 and 1.106; thus the mean titre of the geriatric patients was 14.9-fold. Six hours after the injection penicillin was found in all the sera of the geriatric group but in none of the controls.

The higher serum concentrations of the geriatric patients are evidently not due to a slower resorption of the penicillin given as the half-hour means for the geriatric patients are higher than for the control group. Nor, apparently, is there any correlation between

the patient's weight and the serum concentrations, as Savolainen and Tommila also established in their investigations by the same technique (though with procaine penicillin) (4). The weaker secretion by the kidneys (also without clinical symptoms of renal insufficiency) has been considered the principal reason for the higher penicillin blood levels of older patients (2, 5). As the excretion of penicillin occurs mainly through the active function of the tubuli, this conclusion supports the general idea of a weakening of active secretive function with advancing age. In the present experiments the number of penicillin units excreted in the urine of the younger group is greater than that of the older, but the mean penicillin concentration per ml of urine is higher for the aged. Possible differences in the fluid balance and the diffusion of penicillin in the other fluids of the body, which might affect the serum concentrations, were disregarded in the present instance. Due to the limited and clinically heterogeneous material the reported tests must be considered preliminary. In considering penicillin blood levels extrarenal factors must also be borne in mind.

SUMMARY

Nine geriatric patients and nine control subjects were given 200,000 units of water-soluble G-penicillin intramuscularly. The serum concentrations of the geriatric patients were found to be considerably higher: the mean values after 3 hours were almost 15-fold, and after 6 hours, when none of the control subjects showed any demonstrable penicillin in the blood, the aged had 0.2 units of penicillin per ml.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, FROM THE FIRST MEDICAL DEPARTMENT, UNIVERSITY OF HELSINKI, AND FROM THE LABORATORY OF PATHOLOGY, CENTRAL INSTITUTE FOR RADIOTHERAPY

GLANDULAR TOXOPLASMOSIS

A CASE REPORT

by

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(Received for publication February 8, 1955)

Much has been written on toxoplasmosis and toxoplasma since the year 1939, when Wolf, Cowen and Paige (26) reported on the first parasitologically verified case of toxoplasmosis. While the clinical picture of toxoplasmosis congenita can be regarded as distinct and well-defined, there is still much obscurity in that of acquired toxoplasmosis, and the number of parasitologically verified cases of the latter has remained small (23). On the basis of our present knowledge toxoplasmosis aquisita can be divided into the following four types: (1) the exanthematic form (8, 12); (2) the encephalitic form (4, 13); (3) an ocular form without any other systemic manifestations (11, 25); (4) a glandular form accompanied with rise of body temperature, lymphocytosis and atypic leucocytes (resembling those found in mononucleosis infectiosa) and showing a negative Paul-Bunnells reaction. The glandular form has been studied above all by Scandinavian investigators headed by J. Chr. Siim (16). As far as is known Siim was the first to succeed in isolating toxoplasma from lymphatic gland (16, 18). Later on he managed to isolate toxoplasma in six cases of glandular toxoplasmosis out of seven (19). Siim (17) has described the microscopic picture of a biopsy of lymphatic gland in one of his cases as follows: »the lymph node showed pathological changes with pronounced hyperplasia of the reticulum cells in the follicular center, with numerous mitosis

and intensively stained well-defined granulas of varying shape and size.»

Besides, other investigators including Gard & Magnusson 1950, Magnusson, 1950, Wahlgren 1950, Ström 1950, Landau 1951, Bang 1953 and Jacobson 1953 have dealt with the adenopathy of toxoplasma.

According to Wahlgren (24) the histological picture of the lymphatic gland has shown remarkable similarity in all cases investigated by him. The swelling of reticulum cells and an abundance of eosinophilic leucocytes and plasma cells can be regarded to compose a picture so far typical as to be suggestive of toxoplasmosis.

In 1953 Armstrong and MacMurray (1) succeeded in isolating toxoplasma out of a lymph node. It was then inoculated upon white mice, but no signs of illness or toxoplasma could be detected until after 137 days, which is a relatively long time, particularly with view to the fact that white mice are infected under laboratory conditions (10).

Stanton and Pinkerton (21) found a pseudocyst in a histological section removed from a lymph node. Basing on the findings of Scandinavian investigators and supplementing them with their own work, these two investigators came to the conclusion that in the glandular form of toxoplasmosis the histological picture is sufficiently typical to lead to diagnosis. According to them the histological picture shows: »1) small groups of swollen histiocytes scattered throughout the reticuloendothelial stroma and not confined to usual sinus pattern; 2) multinucleated giant cells associated with, and apparently formed by fusion of these histiocytes; 3) marked mitotic activity of the reticulum cells without marked distortion of the follicular architecture; and 4) phagocytosis of large amounts of swollen eosinophilic cells into separate clumps throughout the lymphoid architecture suggesting diminutive sarcoid lesions.»

Skipper et al. (20) reported 3 cases of acquired toxoplasmosis. Two of these cases simulated glandular fever. The patients had dye-test titers of 1/8000 and 1/9000 and complement fixing antibodies in a dilution of 1/160 and 1/64.

Cathie (2) reported a case of adenopathy in a child. Dye-test 1/1024 and complementfixing antibodies 1/128. It was possible to isolate toxoplasma from the blood and saliva of this patient.

Since 1951 one of us (PG) has been conducting a routine investigation on all sera sent in for Paul-Bunnells reaction. This material includes one case with exceptionally high dye-test titre and high complement-fixation titre. In the following we shall give a detailed description of this case.

CASE REPORT

E.T. Farmer's wife from Central Finland. Age: 22 years. Mother had been under treatment for tuberculosis two years ago. Three years ago the patient had given birth to a healthy boy. At home her work consisted of household tasks and care of the live stock. There had been no sick animals in the house. About six months ago she had felt tremor in upper and lower extremities, which had continued to trouble her. There had been periodic rise of temperature ad T ax 38°C. during one month. She had been administered penicillin with the result that temperature had returned to normal within one week. At the same time she had noted the appearance of tender glands on her neck and under her right arm. These had however grown smaller and lost their tenderness right before admission to the hospital.

The patient's general condition was good and she looked healthy. T ax 36.9—37.3. Pulse frequency was 70/min. Liver and spleen were of normal size. Two pea-sized lymph glands were observed on the right under the chin and on her neck there was one gland of the size of a prune. Furthermore, there were two prune-sized glands under her right and left arms. The latter were solid, showed no tenderness and moved freely under the skin. There were no scars of ulceration, no neurological symptoms and no tremor.

A laboratory examination gave among others the following results: SR: 22—13—15 in specimens taken at intervals of one week. In blood count the number of leucocytes was found to be normal at the beginning; lymphocytosis 61.5 per cent, but returned to normal within one month. Red blood count was normal. Bonemarrow specimen normal. In electrophoresis gamma-globulin values were 28% (normal value 15%). Widal negative. Paul-Bunnell reaction 1/8 to 1/16. AST 320—400. X-ray examinations revealed nothing out of the normal in the lungs and in the heart.

A biopsy was taken from the patient's neck and subjected to histological examination. The patient was given a Streptomycin-PAS-INH treatment for three weeks, during which time she was administered a total of 19 g. Streptomycin, 70 g. PAS and 3 g. Rimiphon. No change could be observed in the size of the glands. After one month's stay at home the patient was readmitted to hospital. On admission her status was unchanged. Dye-test titres observed in connection with Paul-Bunnell's reaction were extremely high. A new specimen was taken from the axillary lymphglands. Besides a muscular biopsy was taken from the patient's calf.

The patient was given 4 g. of sulphamerazin per day for a period of 20 days, during which time toxoplasma titres were recorded. (See table 1.)

An inquiry after the patient's health nine months later revealed that her health was good. The dye-test titre of the last specimen was 1/256 and the complement-fixation titre 1/4.

Histology (E. S.). — The normal architecture of the lymph node was preserved. Instead, signs of strong and diffuse reticulosis were noted therein. Germinal centres were wide, loose and misformed and their limits were frequently vague. They showed an abundance of mitosis and particles staining like chromatin and having here and there a »new moon» configuration. No atypic mitosis or necrosis could be observed. An uncommon feature was the appearance of swollen histiocytes throughout the reticuloendothelial stroma (Figures 1 and 2). They were frequently found in small groups of 5 to 10 cells. No distinct giant cells were observed, though cell-boundaries were often rather diffuse. The nuclei were hypochromatic and oval, and one prominent nucleolus could be discerned in them. Cytoplasm was eosinophilic.

The structure of the lymph node was fully compatible with what has been written on changes in connection with toxoplasmosis in the literature.

No distinct giant cells were noted in this special case, but in the histiocyte collections cell-boundaries were extremely vague. Stainings for bacteria and fungis were negative and no micro-organisms could be detected in the slides. No pseudocysts described in the literature were noted.

The preparations were sent to J. Chr. Siim who writes: »Though not acquainted with the serological reaction I should say that the findings are identical with what we have found in our cases of toxoplasmosis acquisita with lymph gland swelling, and in which we have succeeded in isolating toxoplasma.»

Laboratory Diagnosis. — 17. 3. 1954. One half of a lymph node extirpated from the inguinal fold of the patient, was ground in a mortar together with 2 ml of physiological sodium chloride solution. The suspension was injected intraperitoneally on six white mice. The mice were kept under daily observation. After four weeks blind passage was performed on four of the mice. Parts of their

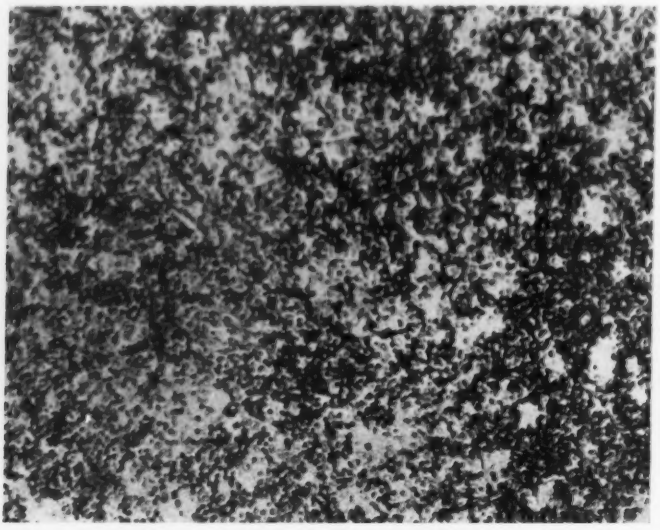

Fig. 1. — Small groups of swollen histiocytes scattered throughout the reticulo-endothelial stroma. Htx. eosin × 150.

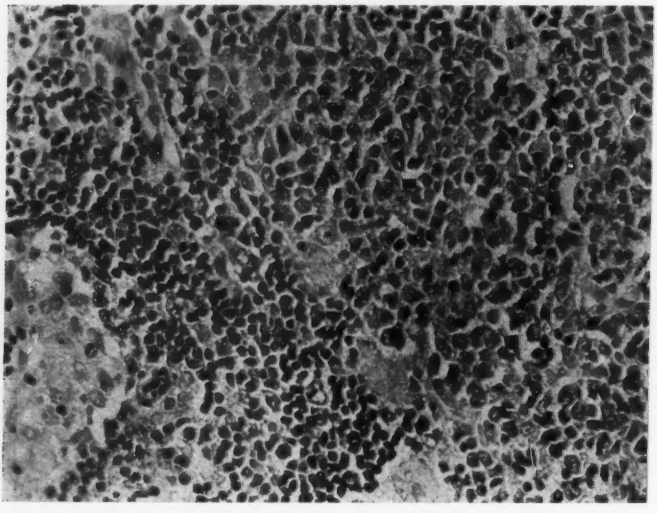

Fig. 2. — From the margin of a germinal center. The cell proliferation and the pale histiocytes are clearly visible. Htx. eosin × 300.

brain and liver were ground as above and the suspension injected intraperitoneally on new mice. It is possible that the interval between the blind passages was too long. A »klatsch-preparation» was made of the liver and brain of each mouse. Formations resembling toxoplasma were noted in some of these preparations, but it was impossible to ascertain with certainty whether it actually was toxoplasma. Although a total of six such one month's passages were performed, no toxoplasma could be isolated with certainty.

Dye-tests and complement-fixation tests were repeated several times during illness. Results are shown in Table 1.

TABLE 1

Date	Dye-test	Complement-fixation reaction
1. 2. 54	16,384 [1]	32
12. 2. 54	16,384	32
16. 3. 54	16,384	128
22. 3. 54 [2]	65,536 [1]	128
29. 3. 54 [2]	65,536 [1]	256
2. 4. 54 [2]	65,536 [1]	512
7. 4. 54 [2]	16,384	512
10. 4. 54	16,384	512
13. 4. 54	—	512
23. 4. 54	—	128
11. 5. 54	65,384 [1]	64
17. 1. 55	256	4

[1] = Results were controlled three times.
[2] 4.0 gr of sulpha per day.

The table gives the inverse values of titre figures. In dye-test the method recommended by Sabin & al. (14) in 1952 was strictly followed. Accordingly, the titre figures denote the dilution of the serum before addition of accessory factor and toxoplasma suspension. The pipette was changed for every tube after dilution 1/256.

The antigen for the complement-fixation reaction was made of chick chorioallantois membrane in accordance with Warren and Russ. The antigen was high speed centrifugalized according to Sabin.

DISCUSSION

In the case described in the foregoing it was not possible to isolate toxoplasma from the lymph gland tissue.

Though a low dye-test titre may be unspecific (6), on the other hand a dye-test titre as high as the one observed in this special case (1/65,536) and — in our opinion — particularly the high complement-fixation titre (1/512) can be regarded as suggestive of toxoplasmosis. The parasitologically verified case of toxoplasmosis describea by Kass & al. (8) had a dye-test titre of 1/32,768 on the 73rd day of illness, but in this case an abundance of toxoplasms were observed throughout the whole body. E.g. autopsy revealed the existence of a great amount of toxoplasms in different muscle tissues. In our case no pathological changes could be detected in the muscle biopsy.

Considering the specifity of pathological changes in the lymph nodes, the case was first suspected to be one of lymphogranulomatosis maligna. In the second examination (ES) the diagnosis was reticulosis with anote that it is peculiar and might be mononucleosis infectiosa. After having studied changes described in connection with toxoplasmosis, the pathologist has suggested toxoplasmosis on the basis of lymph node specimens in two other cases one of which showed the titre of 1/16,384 and the other 1/4096.

On account of the foregoing it is evident that the picture can be suggestive of malignant lymphoid tumour, and that in case the investigator is familiar with changes found in toxoplasmosis, the changes in the lymph nodes may lead him to suspect toxoplasmosis.

The question of the constancy or specifity of these changes has so far remained unsolved. However, it seems apparent that this histological picture is accompanied with high dye-test and complement-fixation titres.

Kass et al. (8) were not able to save their patient with sulpha, aureomycin and sulphon treatment. However, at autopsy no fresh foci could be detected which rather seems to suggest that the treatment had perhaps had some effect on the infection. Our first impression was that sulpha treatment had no effect on the infection. Dye-test titre remeined high. However, this does by no means exclude the possibility that in the long run the sulpha treatment

may, after all, have had a certain effect reflected in the sharp decline of the complementfixation titre. The fact that the dye-test titre rose again after having been 1/16,384 at one time, may have been due to the test technic .Nevertheless, it has to be noted that the same phenomenon was observed by Ström (22) in one of the cases of laboratory infection, with the maximum dye-test titre of 1/4000.

SUMMARY

A case of glandular toxoplasmosis with exceptionally high dye-test titre and high complement-fixation titre is described. The patient is a woman of 22 years of age.

The changes found in lymphglands in the glandular form of toxoplasmosis are so far typical as to be suggestive of toxoplasmosis.

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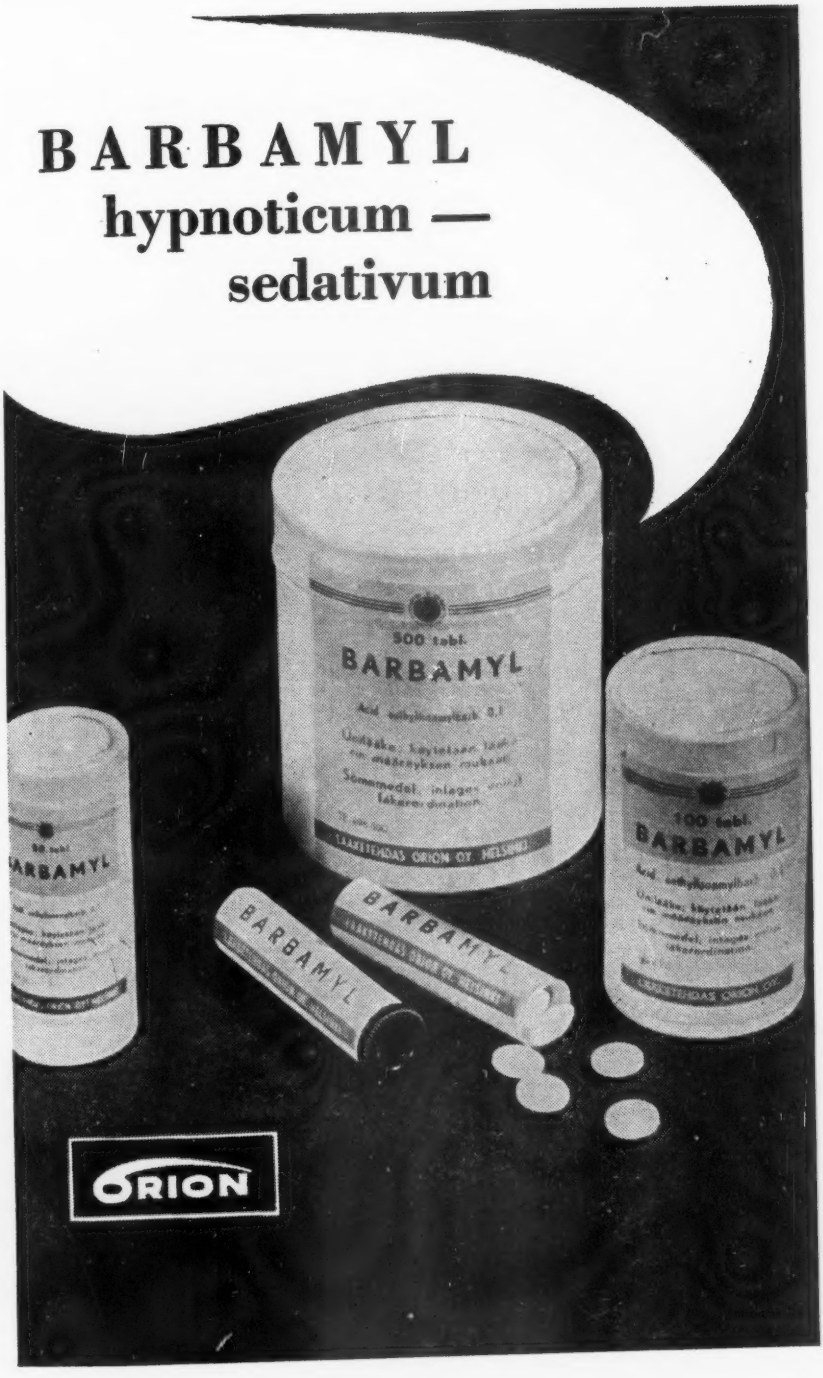
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